The Role of Inflammation and Fibroblasts in Conjunctival Scarring in Ocular Mucous Membrane Pemphigoid

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I, Valerie Ping Jian Saw, confirm that the work presented in this thesis is my own. Where information has been obtained with the help of others, I confirm that this has been indicated below:

1. Two of the 20 antibody array kit experiments investigating matrix metalloproteinase levels in conditioned medium were carried out by Robin Dart and one eighth of a kit was carried out by Temi Miade (Chapter 4, sections 4.3.6 and 4.4.3). The densitometry measurements and statistical analysis were carried out by me.

2. Three of the six experiments and analysis investigating surface expression of activation markers and costimulatory molecules by conjunctival fibroblasts in response to interferon-gamma (IFNγ) (Chapter 5, sections 5.3.9 and 5.4.5) and were carried out by Robin Dart, under my supervision. The remaining experiments and statistical analysis was carried out by me.

3. The T cell culture supernatant used in the migration assay in Chapter 5, sections 5.3.8 and 5.4.4 was obtained from experiments carried out by Eu Lee and Ifeoma Offiah.

4. One of the four experiments investigating chemokine expression by pemphigoid conjunctival fibroblasts (Chapter 6, sections 6.3.8 and 6.4.8) was carried out by Ifeoma Offiah, and one of the chemokine bead arrays was carried out by Grazyna Galatowicz.

Abstract

Ocular mucous membrane pemphigoid (ocular MMP) is a visually devastating disease where up to 30% of patients become blind due to the consequences of conjunctival inflammation and aggressive fibrosis. Standard systemic immunosuppression controls inflammation, but is limited by toxicity. How well immunosuppressive therapy prevents fibrosis is unknown. The cellular and molecular mechanisms that lead to excessive conjunctival fibrosis in ocular MMP are incompletely understood. Chronic inflammation and repair play an important role, but the conjunctival fibroblasts may also be autonomously activated.

This thesis aimed to investigate potential mechanisms involved in conjunctival fibrosis in ocular MMP, for the purpose of proposing future anti-fibrotic therapies, to be used in conjunction with systemic immunosuppression.

A retrospective review established that whilst conjunctival inflammation appeared to be controlled in 70% of patients, fibrosis progressed in 53%. A pilot randomised trial of adjunctive pulse intravenous methylprednisolone in patients with severe ocular MMP commencing both oral cyclophosphamide and oral corticosteroids did not reduce the time to control of inflammation, which had been hypothesised to prevent the progression of scarring.

Both tumour necrosis factor-alpha (TNFα) and the T cell-derived fibrogenic cytokine interleukin-13 (IL-13) were expressed in active ocular MMP. Although this expression was reduced after treatment in clinically uninflamed ocular MMP, it was still significantly elevated compared to normal conjunctiva. Both TNFα and IL-13 stimulated migration and altered matrix metalloproteinase expression by normal conjunctival fibroblasts. IL-13 also stimulated collagen contraction. Both TNFα and IL-13 upregulated surface expression of costimulatory molecules by conjunctival

fibroblasts, which suggests facilitation of a potential mechanism for fibroblast-T cell cross talk.

Finally, pemphigoid conjunctival fibroblasts showed an altered phenotype compared to normal conjunctival fibroblasts, with increased cell division, migration, collagen contraction, type I collagen secretion, and secretion of eotaxin and matrix metalloproteinase-3.

This thesis increases our understanding of the mechanisms involved in conjunctival fibrosis in ocular MMP, and provides new avenues for investigation of potential adjuvant therapies which could improve the prognosis in this poorly understood disease.

Publications arising during the period of the thesis

Ocular mucous membrane pemphigoid- a strategy for diagnosis and management. Saw VPJ, Dart JKG. **The Ocular Surface** 2008;6(3):128-42.

Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes.
Saw VPJ, Dart JKG, Rauz S, Ramsay A, Bunce C, Xing C, Maddison PG, Phillips M. **Ophthalmology** 2008 Feb;115(2):253-261.

Book chapter: Management of ocular mucous membrane pemphigoid.
Saw VPJ, Dart JKG. In: **Essentials in Ophthalmology-Cornea and External Disease**. Editors. Reinhard T, Larkin F. Springer-Verlag, Heidelberg, 2008. Chapter 8, p.154-175.

Manuscript in press

Tumour necrosis factor-α in ocular mucous membrane pemphigoid and its effect on conjunctival fibroblasts.
Saw VPJ, Dart RJC, Galatowicz G, Daniels JT, Dart JKG, Calder VL. **Investigative Ophthalmology and Visual Science.**

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Abbreviation List

APC	antigen presenting cell
BMZ	basement membrane zone
CCL11	chemokine (C-C motif) ligand 11, also known as eotaxin-1
CFSE	carboxyfluorescein diacetate, succinimidyl ester
DIF	direct immunofluorescence microscopy
ECM	extracellular matrix
FCM	fibroblast culture medium (containing 10% serum)
GMA	glycol methacrylate
HLA-DR	human leukocyte antigen- DR
HSP47	heat shock protein 47
IFNγ	interferon-γ
IgG, IgA	immunoglobulin G, immunoglobulin A
IIF	indirect immunofluorescence microscopy
IL-13	interleukin 13
IVMP	intravenous methylprednisolone
TGFβ	transforming growth factor-β
Th1, Th2	type 1 helper T cell, type 2 helper T cell
m-CSF	macrophage-colony stimulating factor
MIF	macrophage migration inhibitory factor
MMP	mucous membrane pemphigoid
mmp	matrix metalloproteinase
NEAA	non-essential amino acids
OCP	ocular cicatricial pemphigoid
PBS	phosphate-buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM-1	platelet/ endothelial cell adhesion molecule-1
PHA	phytohaemagglutinin

SMA	smooth muscle actin
SFM	serum-free medium (containing 0.1% bovine serum albumin)
timp	tissue inhibitor of matrix metalloproteinase
TNFα	tumour necrosis factor-α

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Introduction and overview

Ocular mucous membrane pemphigoid (ocular MMP) is a visually devastating immunobullous disease characterized by conjunctival inflammation and progressive conjunctival fibrosis. It is one of the most difficult external eye diseases to manage and up to 30% of patients become blind. The conjunctival inflammation in ocular MMP causes discomfort, limbitis which leads to corneal stem cell failure, and conjunctival fibrosis. This resulting fibrosis causes additional damage to the external eye including dry eye secondary to lacrimal duct occlusion, conjunctival shortening and scarring, leading to both corneal exposure and inturned lashes. These anatomical changes increase the susceptibility to corneal infection and ulceration, with resultant corneal vascularisation, opacity, keratinisation and ocular surface failure.

Standard treatment of ocular MMP with systemic immunosuppressive therapy controls inflammation, but the effectiveness of systemic immunosuppression in controlling inflammation is limited by its toxicity. There is no reliably effective therapy specific for fibrosis. Furthermore, it is not known how well immunosuppressive therapy prevents fibrosis. The cellular and molecular mechanisms that lead to excessive conjunctival fibrosis in ocular MMP are incompletely understood. In common with many fibrotic disorders, chronic inflammation and repair are believed to play an important role, but it has also been proposed that functional characteristics of the conjunctival fibroblasts may be altered in ocular MMP, similar to the findings in fibroblasts derived from patients with systemic sclerosis.

The aims of this thesis were threefold:

1. To assess how well current conventional immunosuppressive treatment controls inflammation and fibrosis in ocular MMP.

2. To investigate the potential roles of two key inflammatory cytokines, tumour necrosis factor-alpha (TNFα) and interleukin-13 (IL-13), in conjunctival fibrosis due to ocular MMP. These cytokines were chosen for study because they are important mediators of inflammation and scarring in diseases affecting other systems, and inhibitors are either available, or being developed, for therapeutic applications.

3. To investigate whether the phenotype of conjunctival fibroblasts from ocular MMP patients differs from that of normal conjunctival fibroblasts, and how this contributes to conjunctival fibrosis in ocular MMP.

Chapter 1 provides the background to the clinical problem and current understanding of the pathogenesis of conjunctival scarring in ocular MMP.

The next two chapters investigate how well systemic immunosuppressive therapy prevents clinically relevant fibrosis. Chapter 2 is a retrospective review of the clinical outcomes of conventional immunosuppressive treatment, in a large cohort of patients, in terms of both control of inflammation and fibrosis. Chapter 3 describes a prospective randomised controlled trial of adjuvant pulsed intravenous methylprednisolone in severely affected ocular MMP patients commencing maximal immunosuppressive treatment with oral cyclophosphamide and oral corticosteroids. The primary outcome measure of the trial was to evaluate whether the use of adjuvant pulsed intravenous methylprednisolone reduced the time to the control of inflammation, and the secondary outcome measure was to identify whether it reduced the progression of scarring.

Chapters 4 to 6 describe laboratory studies investigating the effect of systemic immunosuppressive treatment on the conjunctiva in MMP at the cellular and molecular level, by comparing conjunctival biopsies taken from eyes with active

inflammation due to ocular MMP, with biopsies taken from clinically uninflamed white eyes where inflammation has been controlled by immunosuppressive treatment.

Chapter 4 describes a study investigating the expression of tumour necrosis factor-alpha (TNFα) in the conjunctiva in ocular MMP in both active disease and in clinically uninflamed eyes following immunosuppressive treatment, and evaluating the effects of TNFα on normal conjunctival fibroblasts *in vitro.* TNFα was selected as the candidate for study in Chapter 4 because, although there are readily available TNFα inhibitors used successfully in other autoimmune inflammatory conditions, the current scientific rationale for their use in ocular MMP, either systemically or as potential local adjuvant therapy, is lacking. There are limited studies investigating the expression of TNFα in MMP tissue. Furthermore, given that TNFα could have either pro-fibrotic or anti-fibrotic effects, inhibition of a molecule with potentially anti-fibrotic effects would be undesirable in the context of ocular MMP.

Chapter 5 similarly investigates whether the key profibrotic mediator interleukin-13 (IL-13) is expressed in the conjunctiva in ocular MMP before and after treatment, and the direct effects of IL-13 on normal conjunctival fibroblasts. Preliminary studies on T cells derived from ocular MMP were carried out, with the intention of investigating the contribution of T cell-fibroblast interactions in ocular MMP. Interleukin-13 was selected for this investigation because previous studies have shown that T cells predominate in the substantia propria during the subacute and chronic phases of ocular MMP. IL-13 is a cytokine secreted by type 2 helper T cells, that plays a key role in orchestrating fibrosis in other settings. It is expressed in mucosal sites during inflammation, and might play an important role in fibrosis in this disease, as it does in liver, lung and colonic fibrosis. IL- 13 inhibitors are also being developed for clinical use.

Chapter 6 investigates whether fibroblast phenotype-dependent mechanisms could be involved in fibrosis in ocular MMP, by evaluating whether conjunctival fibroblasts derived from ocular MMP patients behave autonomously in a profibrotic manner compared with normal conjunctival fibroblasts, as assessed by functional fibroblast assays.

As each chapter deals with a particular aspect of the treatment and pathogenesis of ocular MMP, a review of the literature relevant to that chapter is provided at the beginning of the chapter. Specific methods that were used to obtain the results of each chapter are described within the relevant chapter. These results are then discussed within the context of prior literature. The findings described in the chapters and their significance are then synthesised in the final concluding chapter.

Chapter 1

Background

1.1 Introduction

Mucous membrane pemphigoid (MMP) is part of a spectrum of a systemic autoimmune subepithelial blistering disease, where inflammation and scarring are the distinguishing features. Scarring is responsible for the blinding complications of MMP when it affects the eyes (ocular MMP). Immunosuppressive therapy to control inflammation is the present standard treatment. Current knowledge of the cellular and cytokine interactions that lead to fibrosis in ocular MMP are summarised here. The rationales for selecting TNFα and IL-13 for investigation in ocular MMP are also explained.

1.2 Mucous membrane pemphigoid

Mucous membrane pemphigoid (MMP), previously known as cicatricial pemphigoid (CP) describes a heterogeneous group of immunobullous diseases characterised by recurrent subepithelial blistering of mucous membranes and sometimes skin, where the clinical hallmark is healing of lesions with excessive scar tissue formation. Mucous membranes which can be affected in varying combinations include the mouth, conjunctiva, pharynx, larynx, oesophagus, nose, genitalia and rectum. In some patients, clinical disease is confined to the mouth (oral MMP) or the eye (ocular MMP). MMP blisters affecting the mouth and scalp are illustrated in **Figure 1.1**.

MMP is an uncommon condition, with an incidence of 1.16 per million population per year (Bernard *et al.*, 1995). No racial or geographic predilection has been reported. The female: male ratio is 2:1 (Foster, 1986). Although the average age of onset is 65 years, it can occur in children and young adults (Cheng *et al.*, 2001).

Autoantibodies against epithelial basement membrane zone (BMZ) components are believed to play a role in the pathogenesis of this group of diseases (Oyama *et al.*, 2006). Whilst circulating autoantibodies in a given patient tend to target a single

antigen, autoantibodies from MMP patients with similar clinical phenotypes may target different autoantigens, and the heterogeneity of clinical presentations and diversity of target autoantigens make it difficult to characterise this condition immunologically (Chan *et al.*, 2002).

The problem of heterogeneity of both clinical features and autoantigen targets is further complicated by the fact that in patients with pure ocular disease, the frequency of detection of autoantibodies binding to the BMZ of salt-split skin by indirect immunufluorescence microscopy (IIF) s often low (7%) (Chan *et al.*, 1993) although others have reported weak low titre binding in up to 91% (Bhol *et al.*, 1996). In comparison, autoantibodies binding to salt-split skin can be detected in 50-84% of patients with mouth and skin MMP (Schmidt *et al.*, 2001; Setterfield *et al.*, 1998), and additional immunoblotting techniques on sera from patients with a similar phenotype can increase the sensitivity to 100% (Schmidt *et al.*, 2001). When conjunctiva is used as the substrate for detection of circulating antibodies, antibodies can be detected in up to 50% of patients with ocular involvement (Leonard *et al.*, 1988), suggesting that conjunctival basement membrane contains antigens not present in the skin BMZ. Although some investigators have found a correlation between antibodies binding to β4 integrin in sera from MMP patients with ocular involvement, and α6 integrin in sera from patients with pure oral MMP (Rashid *et al.*, 2006), these findings have not been easily replicated by other groups, possibly due to the method used to demonstrate the presence of binding antibodies. Another group have identified IgA antibodies reacting specifically with a 45kD protein in patients with pure ocular MMP, but the nature of this protein has not been elucidated as yet (Smith *et al.*, 1993). These findings suggest that pure ocular disease, and pure oral disease, may be distinct subsets of MMP.

The severity of scarring and functional consequences of scarring in MMP vary depending on the anatomical location affected. Excessive scarring of the oesophagus and larynx can be fatal and requires surgical intervention. In contrast,

lesions of the mouth frequently do not cause scarring. The reason for the widely differing amounts of scarring according to location is unclear, but could relate to differences in the inflammatory response of the tissues, and site-specific characteristics of the fibroblasts (Flavell *et al.*, 2008). It has been noted that the mouth is much less prone to scar formation in response to injury, and that this correlates with its reduced inflammatory profile (Szpaderska *et al.*, 2003). Specific localization of the target antigen in MMP in deeper layers of the BMZ (Lee *et al.*, 2003) has also been postulated as a reason for the tendency to heal with scarring. The focus of this thesis is MMP affecting the eye, where the scarring response can be severe and aggressive, and the functional consequences devastating, with permanent blindness resulting in 27% of those with ocular involvement (Hardy *et al.*, 1971).

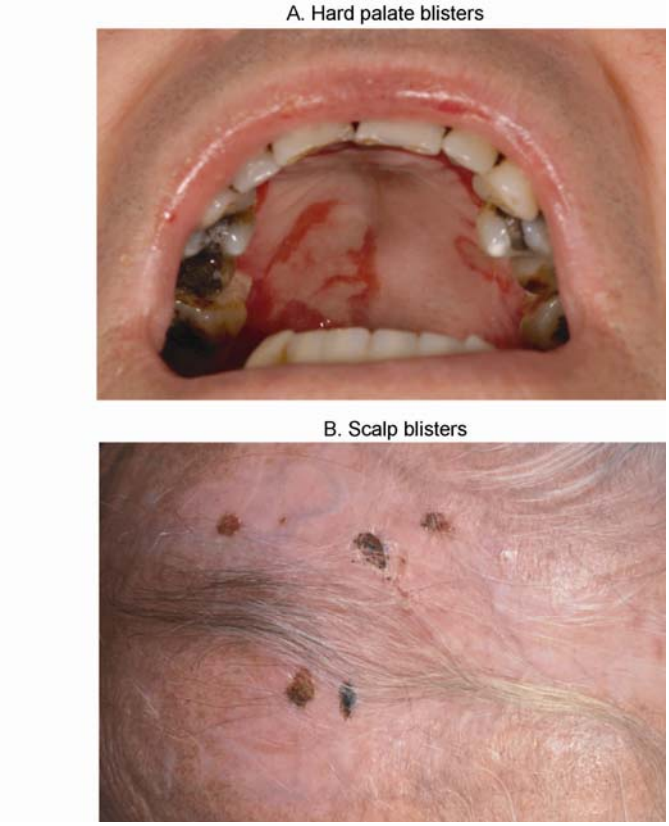


Figure 1.1 Mucous membrane pemphigoid blisters affecting the mouth and scalp

1.3 Ocular mucous membrane pemphigoid (ocular MMP): clinical presentation

As the focus of this thesis is MMP with ocular involvement, henceforward this review will be limited to discussion of current knowledge and understanding about patients with “ocular MMP”, which includes both patients with isolated pure ocular MMP, and MMP patients with ocular plus extraocular involvement. Where evidence has been described as having been obtained from MMP patients without ocular involvement (“extraocular MMP”), or from patients with isolated pure ocular MMP, this is stated in the text. Many studies on MMP with ocular involvement do not segregate results from patients with pure ocular disease, from those with ocular plus extraocular disease.

1.3.1 Anatomy of the conjunctiva

Ocular MMP affects the external eye, which consists of the cornea, conjunctiva, limbus, and eyelids. The **cornea** is the transparent, avascular dome-shaped window covering the front of the eye, that provides 2/3 of the eye’s focusing power. Vascularisation of the cornea leads to blindness due to permanent scarring and irregular astigmatism. The **limbus** is the transition zone between the cornea and sclera. It is the site where stem cells of the corneal epithelium reside. Damage to these cells leads to limbal stem cell failure, where healthy clear corneal epithelium is not able to regenerate, and instead the surface of the cornea is covered with opaque vascularised conjunctival epithelium. The **conjunctiva** is a thin, translucent mucous membrane that derives its name from the fact that it attaches the eyeball to the eyelids. It consists of a superficial epithelium which varies in structure depending on location, from a stratified squamous non-keratinizing epithelium (close to the lid margin) to a stratified columnar epithelium (bulbar). The conjunctival epithelium consists of 2 to 7 layers of epithelial cells, and includes other cell types: goblet cells, melanocytes and Langerhans’ cells. The conjunctival epithelium is continuous with the corneal epithelium at the limbus, and with the skin at the lid margin. Underlying the conjunctival epithelium is a loose vascular

connective tissue stroma called the **lamina propria**. The lamina propria has a looser lymphoid layer above, and a deeper fibrous layer. In a healthy eye the lamina propria contains immunocompetent cells, including mast cells, eosinophils, plasma cells, and lymphocytes, forming diffuse or discrete subepithelial aggregates called conjunctiva-associated lymphoid tissue (CALT). Below the lamina propria lies the fascia bulbi (also known as Tenon's capsule), under which lies the episclera and the scleral wall of the eye.

The conjunctiva is reflected from the anterior portion of the sclera at the superior and inferior fornices, onto the tarsal surface of the eyelids, forming a potential sac, the conjunctival sac. The conjunctiva can be described as consisting of 3 main regions: (1) **Tarsal (palpebral) conjunctiva** lines the inner surface of the eyelids and is tightly bound to the tarsal plate; the subepithelial connective tissue stroma is thin in this region. (2) **Forniceal conjunctiva** lines the superior and inferior conjunctival fornices and are continuous at the medial and lateral canthi. The ducts of the lacrimal glands producing aqueous tears empty into the superolateral fornix. (3) **Bulbar conjunctiva** clothes the anterior part of the eyeball, including the extraocular muscle insertions and Tenon's capsule. **Figure 1.2** shows the macroscopic and microscopic anatomy of the conjunctiva.

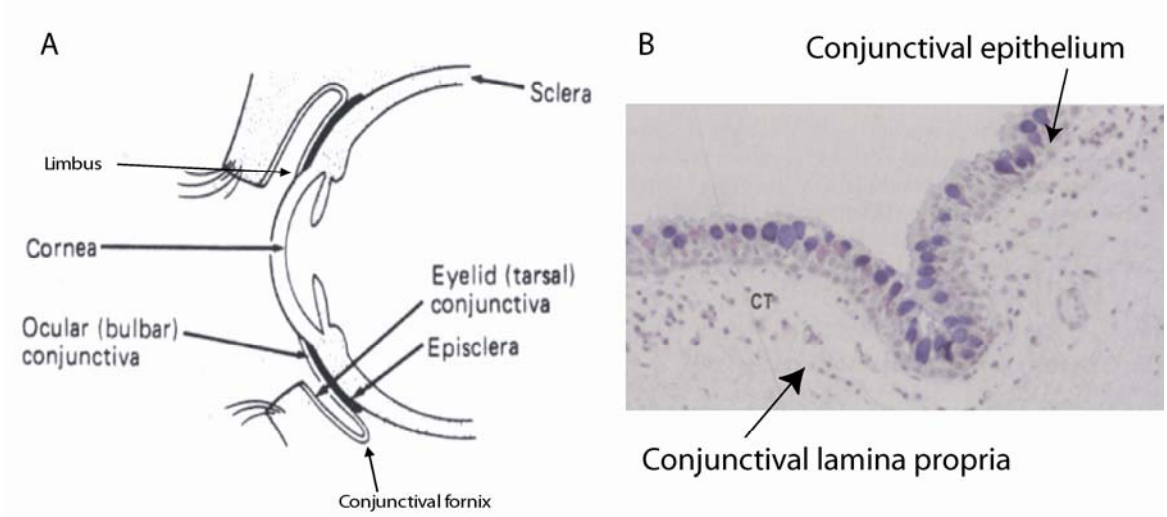


Figure 1.2 Anatomy of the conjunctiva. A. Macroscopic: The conjunctiva connects the eyelid to the eyeball and is composed of tarsal, forniceal and bulbar conjunctiva. The conjunctiva is continuous with the cornea at the limbus. **B.** Microscopic: The conjunctiva is composed of stratified columnar epithelium, 2 to 7 cells thick, overlying a loose vascular connective tissue (CT) layer called the lamina propria.

1.3.2 Clinical presentation of ocular MMP

Ocular involvement in MMP, also known as ocular cicatricial pemphigoid (OCP), occurs in 70% of MMP patients (Hardy *et al.*, 1971). Extraocular manifestations of MMP are present in 50% of ocular MMP patients (Saw *et al.*, 2008). Ocular MMP typically presents with a red eye and persistent conjunctivitis that has not responded to topical therapy, or with recurrent entropion (inturned eyelid) and trichiasis (eyelashes abrading the globe), sometimes following surgical repair. It can also present with ptosis (droopy upper eyelid). About 10% of patients present with acute disease, manifested by acute conjunctivitis and limbitis (inflammation and oedema along the corneoscleral limbus) (**Figure 1.3 A,B**) which leads to rapidly progressive scarring and ocular surface failure (Saw & Dart, 2008a) (**Figure 1.3 C,D**). Unlike other sites of MMP involvement, conjunctival blisters or ulcers are infrequently observed; acute sectoral or diffuse conjunctivitis are more typical in

acute disease. Nevertheless, the majority of patients (90%) present with subacute or low-grade chronic inflammation and slowly progressive scarring. Because the diagnosis is often delayed, symblepharon (bands of conjunctival fibrosis extending across from the globe to the conjunctival fornix or lid margin) and shortened fornices are usually present when the disease is first recognized.

In patients with subacute disease, the earliest clinical sign in patients is medial canthal scarring, with loss of the plica and caruncle. Linear scarring along the marginal sulcus in the upper tarsal plate is sometimes present early in the disease. Other signs, in order of progression, are subepithelial fibrosis (**Figure 1.4** Stage I), shortening of the conjunctival fornices (**Figure 1.4** Stage II), symblepharon (**Figure 1.4** Stage III) and cicatricial entropion followed by ankyloblepharon (complete adhesion between globe and eyelid margin) (**Figure 1.4** Stage IV). End stage disease is characterised by advanced conjunctival scarring with ankyloblepharon, severe dry eye due to obliteration of the lacrimal ductules, ocular surface failure and blindness due to keratopathy (corneal damage). The modified Foster staging of ocular mucous membrane pemphigoid, as proposed by Tauber et al (Tauber *et al.*, 1992), is shown in **Figure 1.4**.

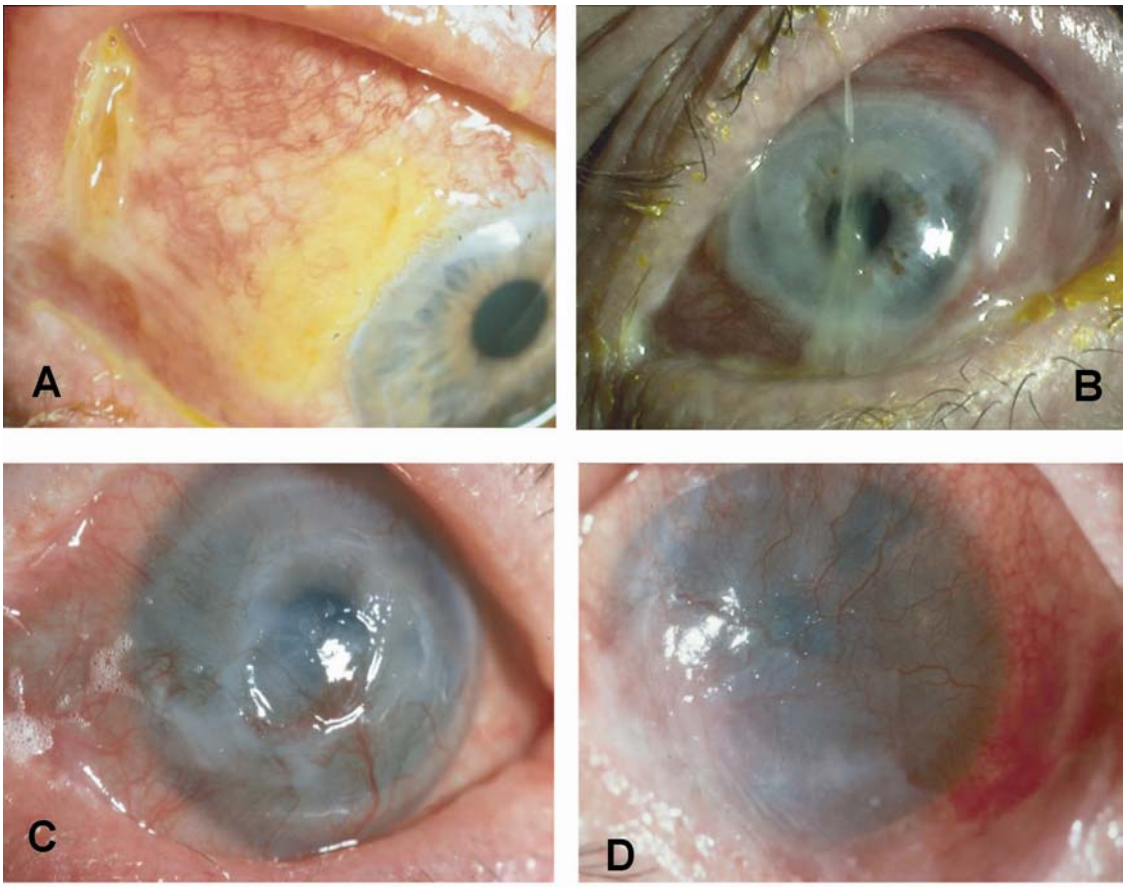


Figure 1.3 Acute onset ocular MMP. A. Conjunctival ulcer demonstrated by yellow fluorescein dye staining in acute ulcerative conjunctivitis. Such ulcers are infrequently seen. **B.** Limbitis (inflammation and oedema of the corneoscleral limbus) from 3 to 4 o'clock indicating inflammatory insult to limbal stem cells which regenerate the cornea with healthy clear epithelium.
C. Rapid progression of conjunctival scarring in an acutely inflamed eye.
D. Ocular surface failure as a sequela of acute uncontrolled limbitis which has damaged limbal epithelial stem cells, resulting in limbal stem cell failure and an opaque, keratinized corneal epithelium with associated vascularisation.

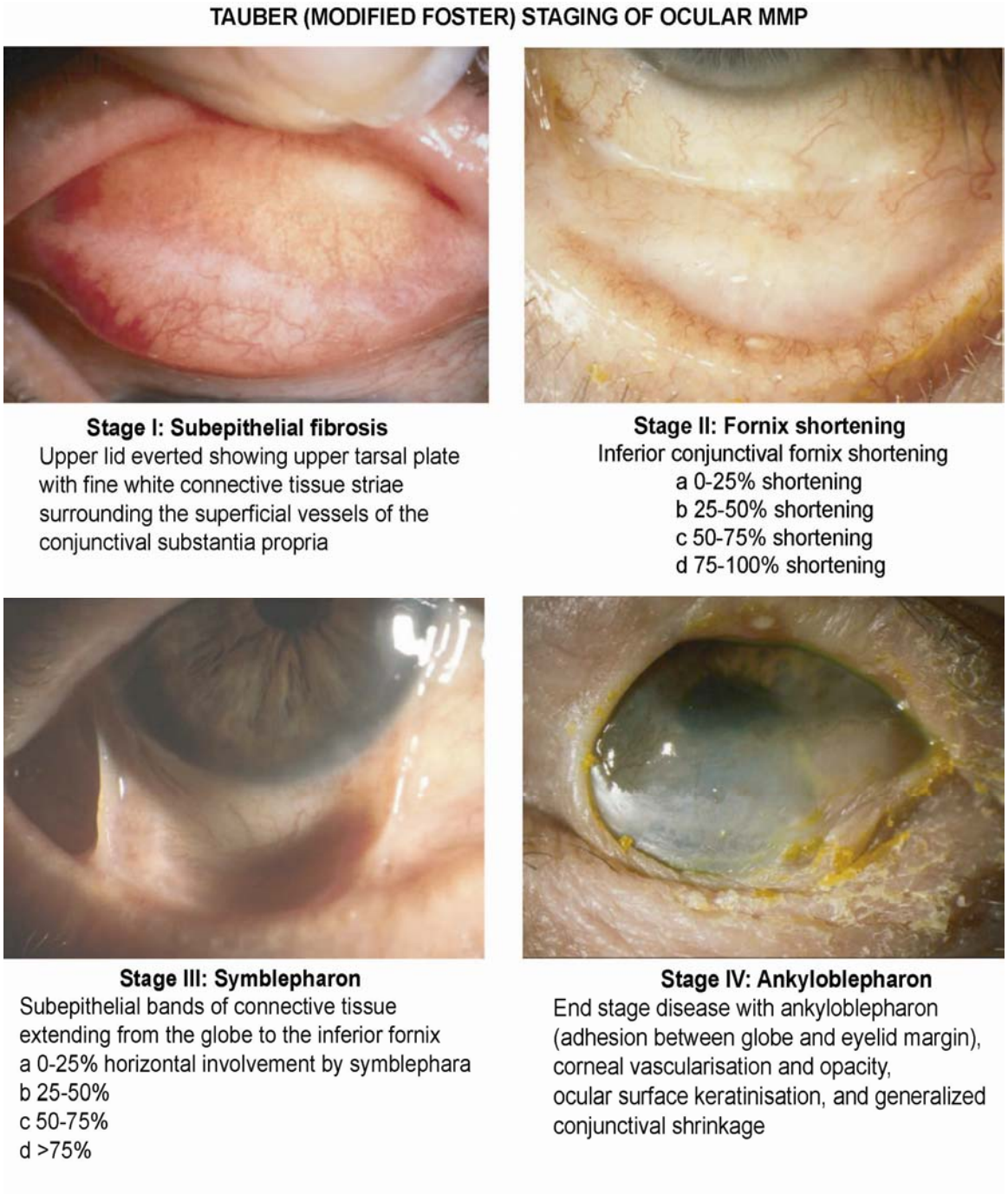


Figure 1.4 Tauber (modified Foster) staging of ocular mucous membrane pemphigoid (Tauber *et al.*, 1992).

1.4 Ocular mucous membrane pemphigoid: treatment

1.4.1 Principles and goals of treatment of ocular disease

The primary goals of treatment of ocular MMP are to control inflammation and arrest fibrosis, in order to prevent progression of disease to more advanced stages and blindness. Optimal management of inflammation in ocular MMP targets the inflammation due to systemic immunodysregulation with immunosuppressive agents, after treating inflammation due to local surface disease (blepharitis, trichiasis, dry eye), infection and toxicity (Saw & Dart, 2008b). Systemic immunosuppression is necessary to control inflammation, as there is no evidence that topical or subconjunctival corticosteroid therapy alters the natural history of the disease (Foster, 1986; Mondino *et al.*, 1979). Most cicatrisation is believed to occur during active inflammation (Mondino *et al.*, 1979), but the efficacy of systemic immunosuppression in preventing fibrosis has not been established. Without treatment, conjunctival scarring in ocular MMP progresses in 64% of patients over 10 to 53 months (Mondino & Brown, 1981). Progression is more frequent in the advanced stages of disease (Mondino & Brown, 1983). No current therapy is able to reverse the cicatrisation or ocular surface problems once they have developed.

1.4.2 Immunosuppressive therapy

Evidence for the use of current immunosuppressive therapy in ocular MMP comes from cohort studies (Foster *et al.*, 1982; Mondino, 1990), interventional and retrospective case series (Doan *et al.*, 2001; Elder *et al.*, 1995; Elder *et al.*, 1996b; Foster *et al.*, 1992; Letko *et al.*, 2001; McCluskey *et al.*, 2004; Mondino & Brown, 1983; Neumann *et al.*, 1991; Rogers, III *et al.*, 1982; Tauber *et al.*, 1991; Thorne *et al.*, 2008) and two randomized trials (Foster, 1986). These have indicated a role for dapsone, sulfasalazine or sulphapyridine for mild to moderate inflammation; azathioprine or methotrexate for moderate inflammation or disease not responding to sulpha therapy; and cyclophosphamide with a short course of prednisolone for

severely active inflammation. The calcineurin inhibitors ciclosporin and tacrolimus have failed to control inflammation in several studies (Foster *et al.*, 1992; Letko *et al.*, 2001; Neumann *et al.*, 1991), and their use in ocular MMP cannot be recommended on the basis of current evidence. Tetracyline and niacinamide have been reported as safer alternatives to immunosuppression in mild-moderate extraocular and ocular MMP (Dragan *et al.*, 1999; Reiche *et al.*, 1998), and mycophenolate mofetil has been described in small case series to be effective in extraocular MMP (Ingen-Housz-Oro *et al.*, 2005; Megahed *et al.*, 2001) and ocular MMP (Zurdel *et al.*, 2001). The efficacy of the most frequently used immunosuppressive agents in controlling inflammation in ocular and extraocular MMP is summarized in **Table 1.1**.

With current immunosuppressive regimens, progression of cicatrisation has still been observed in 10 to 43% of ocular MMP patients (Elder *et al.*, 1996a; Miserocchi *et al.*, 2002; Mondino & Brown, 1983). How well immunosuppressive therapy controls conjunctival fibrosis in ocular MMP is not clear. Furthermore, there is small subgroup of MMP patients with ocular involvement who appear to have ongoing conjunctival fibrosis without overt clinical signs of inflammation (Elder, 1997c). Despite the absence of clinical signs of inflammation, there may still be significant cellular infiltrate on histological evaluation ("white inflammation") (Bernauer *et al.*, 1993b; Elder *et al.*, 1997). Further systemic immunosuppression with potential systemic toxicity may not necessarily be helpful in these cases, for whom more specific local therapy targeting the cellular infiltrate or fibrogenic process would be ideal.

Table 1.1 Results of studies reporting the efficacy of conventional immunosuppressive agents in controlling inflammation in MMP (mucous membrane pemphigoid) with and without ocular involvement

Immunosuppressive agent	Study	Severity of inflammation	% of patients responding to treatment
Nicotinamide and tetracycline	Reiche et al, 1998	Mild-moderate	63% (n=8) extraocular and ocular MMP
Dapsone	Rogers et al, 1982	Mild-moderate	83% (n=24) extraocular and ocular MMP
	Tauber et al, 1991	Mild-moderate	45% (n=69) ocular MMP
	Foster, 1986	Mild-severe	70% (n=20) ocular MMP
Sulphapyridine	Elder et al, 1996	Mild-moderate	50% (n=20) ocular MMP
Sulfasalazine	Doan et al, 2001	Mild-moderate	45% (n=9) ocular MMP
Azathioprine	Tauber et al, 1991	Mild-moderate	33% (n=11) ocular MMP
Methotrexate	McCluskey et al, 2004	Mild-moderate	83% (n=12) ocular MMP
Cyclophosphamide and steroids	Thorne et al, 2008	Mild, moderate and severe	91% (n=44) ocular MMP
	Elder et al, 1995	Severe	15/19 eyes (79%) ocular MMP
	Foster, 1986	Severe	100% (n=12) ocular MMP
Cyclophosphamide	Tauber et al, 1991	Severe	89% (n=9) ocular MMP
Tacrolimus	Letko et al, 2001	Severe	33% (n= 6) ocular MMP
Ciclosporin	Neumann et al 1991, Foster et al 1992*	Mild-moderate	2/22 patients (9%) ocular MMP
Mycophenolate	Zurdel et al, 2001	Severe	9/10 eyes (90%) ocular MMP

*combined results of two case seriesManagement of patients with recalcitrant disease or intolerance to conventional immunosuppressive therapy

1.4.3 Management of patients with recalcitrant disease or intolerance to conventional immunosuppressive therapy

Alternatives to conventional immunosuppressive therapy have been sought where ocular MMP is resistant to treatment, or in patients who have been unable to tolerate conventional immunosuppressants.

Intravenous immunoglobulin

Severely active ocular MMP resistant to conventional immunosuppressive therapy has been treated with intravenous immunoglobulin (IVIg) by Ahmed et al, who have reported good results in several case series (Foster & Ahmed, 1999; Letko *et al.*, 2004; Sami *et al.*, 2004). A major disadvantage of intravenous immunoglobulin therapy is its dependence on donated blood, an increasingly scarce and expensive resource. In one series, during a national blood shortage, patients received ¼ to 1/3 of previous doses, and every one of the 10 patients relapsed with dose reduction, albeit recovering full control within 4 cycles of full dose IVIg (Foster & Ahmed, 1999). Sudden discontinuation of IVIg therapy in 2 patients resulted in severe recurrence and loss of vision (Sami *et al.*, 2004). This is worrying because it is sometimes not possible for patients to present for therapy depending on emergencies and personal situations. Another disadvantage is the inconvenient and costly treatment regime. An infusion cycle of 4h daily on 3 consecutive days is required every 2 weeks until clinical improvement, then every 3-4 weeks, increasing to 16 week intervals for a total of 25-43 months. The main benefits of IVIg appear to be its minimal side effects and effectiveness in disease refractory to conventional immunosuppression, although the studies are small and not randomised.

Tumour necrosis factor-α antagonists

Case reports of seven patients with MMP resistant to conventional immunosuppressive therapy have been described as improving after receiving the TNFα antagonists etanercept (n=6) or infliximab (n=1): 5 patients had ocular

involvement, 2 patients had pure oral disease (Canizares *et al.*, 2006; Heffernan & Bentley, 2006; John *et al.*, 2007; Prey *et al.*, 2007; Sacher *et al.*, 2002). This treatment was given either alone, or in combination with dapsone or other immunosuppressive therapy. In some cases, continued TNFα antagonist treatment was not necessary after achieving initial disease control, but in many cases weekly etanercept treatment was ongoing at the time of reporting.

However, the scientific rationale for the use of TNFα antagonists in MMP based on present evidence is weak. Although it has been reported that serum levels of TNFα are elevated in MMP compared with normal controls (Lee *et al.*, 1993), there are limited studies examining TNFα expression in MMP tissue. Tissue expression appears to be important because in rheumatoid arthritis, whilst there is no evidence that plasma TNFα levels can predict the clinical response to TNFα antagonists, synovial expression of TNFα appears to be a significant predictor of response to TNFα antagonists (Wijbrandts *et al.*, 2007).

Other biological agents

Successful use of rituximab, a monoclonal antibody directed against CD20-positive B cells, has been reported in one case of MMP with severe ocular and extraocular involvement not responding to conventional immunosuppression (Taverna *et al.*, 2007), and there are preliminary reports of its successful use in 6 other MMP patients (Ross *et al.*, 2009) (n=1), (Schumann *et al.*, 2009) (n=1), (Doan S, et al. IOVS 2006;47:ARVO Abstract 94; n=4). This therapy is likely to be useful in preventing pathogenic autoantibody production by B cells. Further investigation of the efficacy of this therapy in MMP in controlled studies is needed.

Daclizumab, a monoclonal antibody which binds CD25 (Tac subunit) of the human IL-2 receptor which is expressed on activated T lymphocytes, has been used successfully in one patient with ocular MMP (Papaliodis *et al.*, 2003). Its use in ocular MMP has not been investigated further.

Systemic administration of campath-1H (alemtuzumab) a monoclonal antibody against CD52, which is the most prevalent cell surface antigen on lymphocytes, particularly T cells, has been reported to induce long lasting remission in patients with severe refractory noninfectious ocular inflammatory disease (Dick *et al.*, 2000). It has been given systemically by us to one patient with ocular MMP (unpublished data), but was unfortunately unsuccessful in controlling inflammation, and the patient developed atrial fibrillation.

1.4.4 Corticosteroids

Topical steroid treatment is ineffective in controlling progressive ocular MMP, offering only variable symptomatic relief (Hardy *et al.*, 1971; Mondino *et al.*, 1979). Its adverse effects of cataract and glaucoma generally outweigh the benefits. Subconjunctival steroids may be temporarily effective, but relapses occur when the injections are stopped (Mondino *et al.*, 1979), and prolonged use also leads to cataract and glaucoma.

High-dose oral corticosteroids appear to be effective at controlling acute disease activity because of their rapid onset of action (Hardy *et al.*, 1971; Mondino *et al.*, 1979), however the high doses required to sustain disease control (greater than 40mg prednisolone equivalents daily) can lead to severe adverse effects including pneumonia, gastrointestinal haemorrhage and cerebrovascular accidents in this elderly patient population. Disease activity recurs when corticosteroid doses are tapered, and the response can be incomplete in 58% of patients (Foster, 1986). For this reason, oral corticosteroids are useful in acutely inflamed eyes as a short 6 to 12 week course in combination with, and whilst awaiting the onset of effect of, immunosuppressive medication (Chan *et al.*, 2002; Foster *et al.*, 1992).

The use of pulse intravenous methylprednisolone (IVMP) or dexamethasone to control active inflammation is mentioned in the management of ocular and extraocular MMP (McCluskey *et al.*, 2004; Sacher & Hunzelmann, 2005), however

there are no studies evaluating its efficacy specifically in ocular MMP. Equivalent doses of oral prednisolone have been shown to give similar clinical and immunological effects when compared with pulse IVMP in rheumatoid arthritis (Smith *et al.*, 1988).

1.4.5 Antifibrotic therapy

Currently, the only demonstrated means of slowing the progression of scarring is good control of inflammation with systemic immunosuppression. Mondino et al have previously reported success with systemic corticosteroids in suppressing acute disease activity and preventing the rapid shrinkage that accompanies active ocular MMP (Mondino & Brown, 1981). Therapy specifically targeted at fibrosis in MMP is limited. Local therapies for conjunctival scarring would be ideal. Mitomycin C, an alkylating agent which inhibits DNA synthesis and prevents fibroblast proliferation, has been injected subconjunctivally (Donnenfeld *et al.*, 1999) or applied intraoperatively following division of symblephara in patients with ocular MMP (Secchi & Tognon, 1996) but no controlled treatment trials have been carried out. In the Donnenfeld study, absence of progression at a mean of 2 years was observed in 8/9 eyes receiving the subconjunctival mitomycin C injection, and there was no recurrence of symblephara at 19 months. Subconjunctival mitomycin C is not widely used in clinical practice for ocular MMP for several reasons, including variable efficacy, the adverse effect of tissue ischaemia which may affect the success of mucous membrane graft reconstructive surgery, and the potential risk of damaging limbal stem cells. In Donnenfeld's series all eyes receiving subconjunctival mitomycin C were Foster stage 3 with minimal or no inflammation. It may be that giving the injection at an earlier stage, whilst the disease is active, could prevent cicatrisation more effectively.

1.5 The immunopathogenesis of fibrosis in ocular MMP

The immunopathogenesis of fibrosis due to MMP in the eye or in any other site is incompletely understood. Our present understanding of the mechanisms and

pathways involved in fibrosis in MMP is limited because of the lack of animal model studies. The majority of evidence is descriptive, with limited scope for mechanistic insight. A rabbit model of acute anti-basement membrane antibody-mediated conjunctivitis to study ocular MMP has been described (Roat *et al.*, 1990), but has not been utilized in further studies. The review presented here and in the sections following, is focused on current understanding of the pathogenesis of fibrosis in MMP affecting the conjunctiva. Where findings have been obtained from MMP affecting other sites, this is stated in the text.

On the basis of available evidence, the current hypothesis is that an as yet unknown trigger, often in a genetically susceptible individual (Setterfield *et al.*, 2001), provokes loss of tolerance to one or more components of the basement membrane zone (BMZ) (Razzaque *et al.*, 2001). This generates specific B cell clones in germinal centres, which produce circulating IgG and IgA autoantibodies to those basement membrane antigens.

Although the presence of autoantibodies is well established in MMP, the exact mechanism by which the autoantibodies cause subepithelial blistering has not been conclusively demonstrated. It has been proposed that they bind to their target antigens in the basement membrane zone and initiate a type II cytotoxic hypersensitivity reaction, activating the complement cascade to cause subepithelial bulla formation (Elder, 1997b). Evidence for a pathogenic role of autoantibodies in MMP blister formation include the correlation between elevated autoantibody titres and MMP disease activity (Setterfield *et al.*, 1999), and demonstration of *in vitro* conjunctival BMZ separation following incubation with autoantibodies derived from patients with ocular MMP (Chan *et al.*, 1999). Linear C3 complement deposition along the BMZ is characteristic in MMP, indicating complement cascade activity (Chan *et al.*, 2002).

Subepithelial bulla formation results in acute inflammatory infiltration of the substantia propria with neutrophils, macrophages, T lymphocytes and dendritic cells (Bernauer *et al.*, 1993b). Continued T lymphocyte and macrophage proliferation and infiltration results in ongoing subacute inflammation. In ocular MMP, this inflammation stimulates formation of new connective tissue by proliferating fibroblasts. Ongoing fibrosis of the conjunctiva leads to a hostile dry ocular surface susceptible to infection, inturned lashes and eyelids, and damage to limbal stem cells by inflammation and fibrosis leads to ocular surface failure and blinding keratopathy (Elder, 1997a). **Figure 1.5** shows a diagrammatic representation of the proposed immunopathogenesis of fibrosis in ocular MMP, which is described in further detail below.

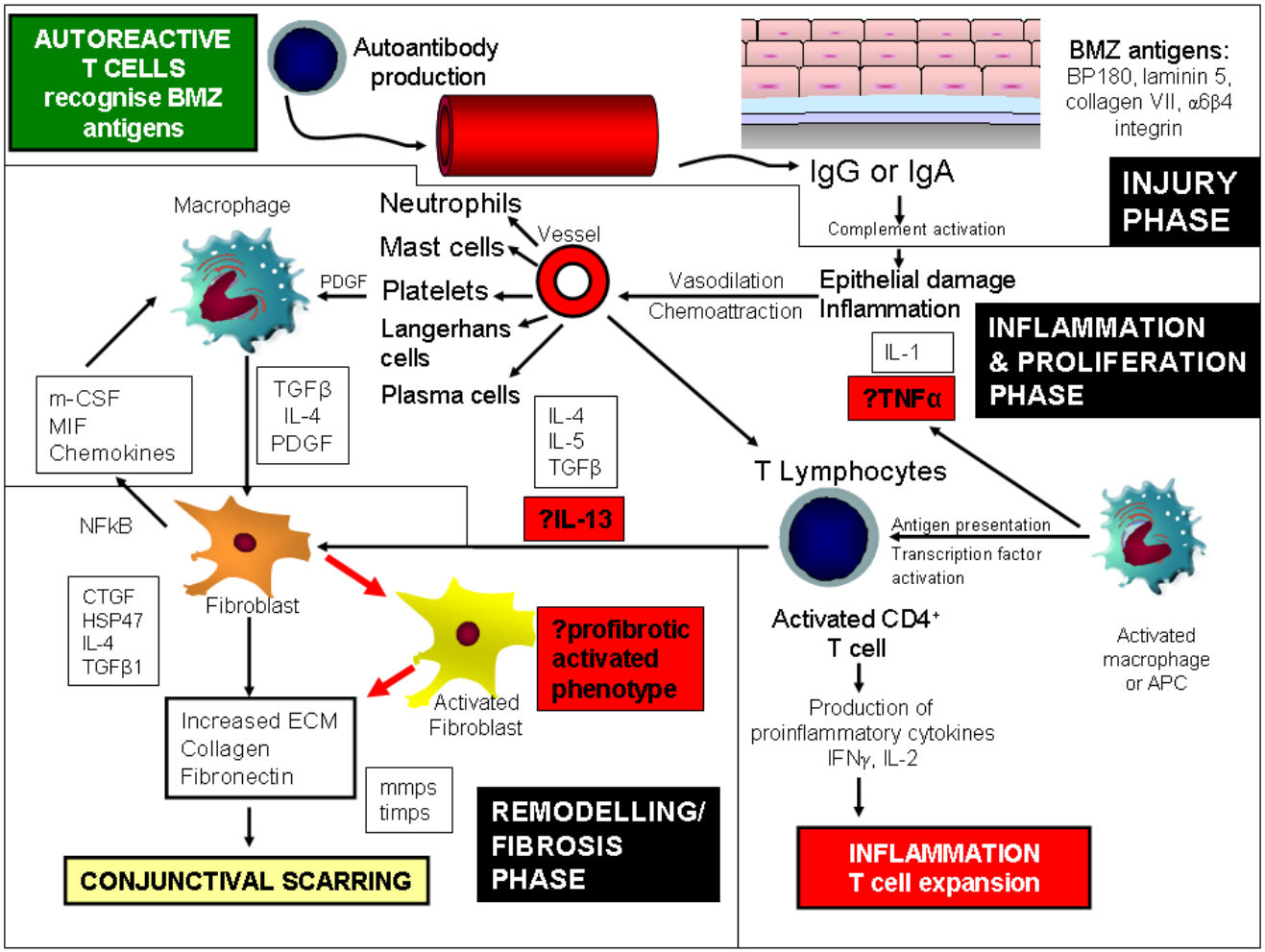


Figure 1.5 Proposed immunopathogenesis of fibrosis in ocular MMP. See next page for detailed legend.The potential roles of TNFα (tumour necrosis factor-alpha), IL-13 (interleukin-13) and profibrotic activated fibroblasts have not been elucidated clearly so far.

Legend to Figure 1.5 Proposed immunopathogenesis of fibrosis in ocular MMP

INJURY PHASE: Autoreactive T cells recognise BMZ (basement membrane zone) antigens (BP180 bullous pemphigoid180kDa antigen, laminin 5, collagen VII, α6β4 integrin) causing B cells in germinal centres to produce autoantibodies IgG (immunoglobulin G) and IgA. These bind to the BMZ and initiate a type II cytotoxic hypersensitivity reaction, activating the complement cascade to cause subepithelial bulla formation.

INFLAMMATION AND PROLIFERATION PHASE: Complement-mediated BMZ, epithelial and connective tissue damage cause vasodilation, release of blood cells and plasma proteins into the damaged site and attracts an acute inflammatory cell infiltrate consisting of neutrophils, activated macrophages, mast cells, platelets, Langerhans cells, and lymphocytes, as well as acute inflammatory cytokine IL-1 (interleukin-1) production. T cell activation and proliferation characteristic of a Th1 (type 1 helper T cell) response occurs, with IFNγ (interferon-gamma) and IL-2 (interleukin-2) production. Th2 (type 2 helper T cell) cytokines IL-4 and IL-5 are also synthesised. Macrophages proliferate and play an important role in scar tissue formation, and also contribute to production of the fibrogenic cytokines TGFβ (transforming growth factor-beta) and PDGF (platelet-derived growth factor).

FIBROSIS PHASE: Fibroblasts become activated, proliferate and synthesise increased extracellular matrix, CTGF (connective tissue growth factor), TGFβ and other cytokines. Endothelial cells may proliferate, forming fibrovascular granulation tissue. The scar tissue is then remodelled, becoming less cellular, and the final result is subconjunctival scarring.

Other abbreviations in **Figure 1.5**: APC antigen presenting cell, m-CSF macrophage-colony stimulating factor, MIF macrophage migration inhibitory factor, NFkB nuclear factor-kappa B, HSP47 heat shock protein 47, ECM extracellular matrix, mmps matrix metalloproteinases, timps tissue inhibitors of matrix metalloproteinases.

Adapted from Elder (Elder, 1997c) and Razzaque (Razzaque *et al.*, 2003a)

1.6 Current knowledge about the inflammatory and fibrotic response in ocular MMP

Unlike tissue remodelling that occurs after an acute non-repetitive injury, for example fibrosis following a chemical or thermal burn, fibrosis associated with chronic repetitive injury, such as that observed in MMP following autoantibody binding to the BMZ, is unique in that the adaptive immune response plays an important role (Wynn, 2004). The persistent inflammatory stimulus leads to lymphocyte-monocyte interactions that sustain the production of growth factors, proteolytic enzymes and fibrogenic cytokines, which together stimulate continued connective tissue deposition.

1.7 The inflammatory cell response in ocular MMP

Immunohistopathology and cellular phenotyping studies have described the inflammatory cellular response in human ocular MMP (Bernauer *et al.*, 1993b; Rice & Foster, 1990; Sacks *et al.*, 1989). Bernauer et al evaluated the histopathological findings according to disease activity, in acute, subacute and chronic disease. These studies provide detailed phenotypic information but limited functional information.

In acute disease, subepithelial bulla formation is accompanied by inflammatory infiltration of the substantia propria by neutrophils, macrophages, dendritic antigen-presenting cells, T lymphocytes and some plasma cells. In acute disease, there is an abundance of neutrophils (42-fold increase) and macrophages (5-fold increase), and a 10-fold increase in T lymphocytes (Bernauer *et al.*, 1993b) in the substantia propria. A relatively high CD4/CD8 ratio (1.0) occurs in acute disease, probably reflecting the role of CD4+ T helper cells in recruiting other inflammatory cells. There is also markedly increased (6-fold) expression of major histocompatibility class (MHC) II molecules (HLA-DR) by fibroblasts, macrophages and lymphocytes,

which indicates the potential for local antigen presentation to T helper cells (Bernauer *et al.*, 1993b). The epithelium in acute disease shows marked infiltration by neutrophils and macrophages, as well as increased numbers of dendritic cells, CD4+ lymphocytes and diffuse HLA-DR expression in some cases (Bernauer *et al.*, 1993b; Rice & Foster, 1990). Other inflammatory cells present at increased levels in the substantia propria include mast cells (Hoang-Xuan *et al.*, 1989; Yao *et al.*, 2003) and a small number of eosinophils (Letko *et al.*, 2002).

During subacute disease in the stroma, the greatest number of T lymphocytes is observed (36-fold increase) and the CD4/CD8 ratio is 0.4 (Bernauer *et al.*, 1993b; Letko *et al.*, 2002). Stromal MHC class II expression is dramatically elevated (10-fold), and, although less elevated than in acute disease, numbers of both macrophages (3-fold) and neutrophils (8-fold) in the stroma are still increased. The epithelium in subacute disease still shows elevated numbers of neutrophils (Bernauer *et al.*, 1993b).

Chronic disease is characterized by cell-mediated immune processes, as evidenced by persistent elevation of T lymphocytes four- (Bernauer *et al.*, 1993b) to 20-fold (Sacks *et al.*, 1989), HLA-DR expressing cells (4-fold), macrophages (3-fold) and dendritic cells (Sacks *et al.*, 1989). The CD4+/CD8+ ratio is 0.5 (Bernauer *et al.*, 1993b). Both Bernauer et al. and Rice et al found only low numbers of B lymphocytes and natural killer (NK) cells at any stage of disease, however Sacks et al found elevated B cell and plasma cell numbers in chronic disease. The epithelium in chronic disease can be normal (Bernauer *et al.*, 1993b) or show increased numbers of T cells and dendritic cells (Sacks *et al.*, 1989).

Clearly, local T lymphocyte proliferation plays a role in acute, subacute and chronic "white" inflammation in MMP. 5-10% of the T cells in all phases of disease express IL-2 receptor (CD25), which in this setting is probably a marker of activation. CD25 can also be a marker for a subset of regulatory T cells ($CD25^{+}CD4^{+}T_{reg}$) (Andre *et*

al., 2009). In active ocular MMP there appears to be a disproportionately increased number of T cells expressing the T cell receptor γ/δ heterodimer, unlike the majority of T cells in normal conjunctiva which express the α/β heterodimer (Soukiasian *et al.*, 1992). γ/δ T cells recognize specific antigens such as heat-shock proteins (HSP), which are known to be elevated in the substantia propria in ocular MMP (Razzaque *et al.*, 2003b). This interaction may be significant in the ongoing autoimmune inflammation observed in ocular MMP.

1.8 The inflammatory mediator and cytokine response in ocular MMP

Inflammatory mediator and cytokine events occurring during the acute phase of ocular MMP are likely to be similar to those described during acute inflammation following complement activation in other clinical settings. Circumstantial evidence suggests a role for several inflammatory mediators and cytokines in acute MMP, based on results of immmunohistochemical staining for protein expression (Bernauer *et al.*, 1993a), in situ hybridization studies of mRNA (messenger RNA) expression (Caproni *et al.*, 2003), and serum cytokine levels (Kumari *et al.*, 2001; Lee *et al.*, 1993).

Although the actual pathways described here have not yet been demonstrated in ocular MMP, they are provided to give an indication of the most likely sequence of events. Activation of the complement cascade results in production of the inflammatory mediators C3a, C5a and bradykinin, increased blood supply, increased vascular permeability, and neutrophil and monocyte chemotaxis (Male & Roitt, 1998). Damaged epithelial and/ or endothelial cells release inflammatory mediators that initiate an antifibrinolytic coagulation cascade, which triggers formation of blood clot and a provisional ECM (Wynn, 2008). Platelets become activated upon exposure to ECM components, and can also be activated by platelet-activating factor (PAF) from neutrophils and macrophages. Degranulation of mast cells and platelets is important in the initiation and development of acute

inflammation, as sources of histamine and 5-hydroxytryptamine which produce vasodilation and increased vascular permeability. Matrix metalloproteinases (mmps) produced by epithelial and /or endothelial cells and activated fibroblasts disrupt the basement membrane and allow recruitment of inflammatory cells like neutrophils and monocytes. Monocytes transform into activated macrophages and release inflammatory mediators including interleukin-1 (IL-1), a multifunctional cytokine playing a key role in inflammation. It is likely that they also release tumour necrosis factor-α (TNFα), another multifunctional acute inflammatory cytokine (see below).

Both IL-1 and TNFα induce activation of macrophages and lymphocytes, increase leukocyte endothelial adhesion and induce lymphocyte secretion of proinflammatory cytokines such as interferon-γ (IFNγ) and IL-2 (Male & Roitt, 1998). They have also been shown to induce the expression of growth factors such as m-CSF (macrophage-colony stimulating factor) and macrophage migration inhibitory factor (MIF) by human conjunctival fibroblasts (Razzaque *et al.*, 2002; Razzaque *et al.*, 2004).

Cytokines and chemokines produced by neutrophils and macrophages are mitogenic and chemotactic for endothelial cells, which help form new blood vessels and granulation tissue. During this period, lymphocytes infiltrate and become activated, and secrete cytokines. The cytokine profile observed in the acute phase of MMP indicates a mixed helper T cell type 1 (Th1) and type 2 (Th2) response. Although the helper T cell populations playing a role in the mucosal immune response in MMP may be more complex than simply Th1 and Th2 subsets, the Th1- Th2 paradigm is useful as an initial framework. Whilst the cellular source of the cytokines has not been confirmed as T cells or other cells in these studies, both IL-2 and IFNγ have been detected in MMP tissues in acute disease suggesting a Th1 response, since T cells are the main source of these two cytokines (Bernauer *et al.*, 1993a; Caproni *et al.*, 2003). IL-4 and IL-5 (Caproni *et al.*, 2003; Razzaque

et al., 2003a) have also been detected, suggesting a Th2 response, although mast cells and other non-T cells can also secrete these cytokines. Elevated serum levels of IL-5 during active MMP have also been reported (Letko *et al.*, 2002). In subacute and chronic disease, IFNγ and IL-2 are less frequently detected (Bernauer *et al.*, 1993a) whilst IL-5, and some IL-4 in the epithelium, is still present albeit at lower levels (Caproni *et al.*, 2003), indicating that a Th2- type response is still present but at reduced levels.

1.8.1 Current evidence for the role of TNFα in ocular MMP

Elevated serum levels of both IL-1α and β (Kumari *et al.*, 2001) and TNFα, but decreased levels of IL-6 (Lee *et al.*, 1993) have been reported in patients with active ocular MMP. There have been 3 small studies investigating the presence of TNFα in MMP tissues: Bernauer et al found abundant TNFα levels in both normal and diseased conjunctiva (Bernauer *et al.*, 1993a); Caproni et al reported moderate focal TNFα expression on the capillary endothelium and on the perivascular infiltrate in submucosal/ dermal tissue in patients with genital (n=2), oral (n=1) and ocular MMP (n=1) (Caproni *et al.*, 1997), and Cordero Coma et al have reported absence of TNFα in normal conjunctiva and prominent TNFα expression in the conjunctival stroma and epithelium of 8 ocular MMP patients (Cordero *et al.*, 2007). None of these studies evaluated the effect of systemic immunosuppressive treatment on tissue expression of TNFα.

1.9 The fibrogenic growth factor and cytokine response in ocular MMP

Profibrotic cytokines and growth factors previously detected in MMP include TGFβ, CTGF, IL-4, IL-5, PDGF, and FGF.

1.9.1 Transforming growth factor-β (TGFβ)

In acute ocular MMP, Elder and Bernauer found increased stromal levels of transforming growth factor (TGF) β1 & β3, which appeared to localize to

macrophages, fibroblasts and plasma cells (Bernauer *et al.*, 1993a; Elder *et al.*, 1997). Caproni et al found similar TGFβ mRNA expression in the lesions of both acute and chronic MMP irrespective of disease activity, and also in bullous pemphigoid, a non-scarring immunobullous disorder. They concluded from this that TGFβ may not play a key role in fibrosis in MMP, but given that the regulation of TGFβ is largely post-transcriptional (Leask & Abraham, 2004), their findings do not exclude a role for TGFβ in fibrosis in MMP.

In subacute ocular MMP, TGFβ, PDGF and FGF are elevated, but in chronic disease these cytokines are only present at low levels (Bernauer *et al.*, 1993a; Elder *et al.*, 1997). This may indicate that once macrophages and fibroblasts are present in large numbers and activated, they become self regulating and remain functionally abnormal after the withdrawal of cytokine influences. On the other hand, it may also indicate that fibrosis occurs predominantly during the acute and subacute low grade inflammatory phases, rather than in chronic, burnt out or inactive disease.

Transforming growth factor (TGF)-β acts on most cell types and stimulates wound healing, fibrosis and matrix production, blocks matrix degradation by proteases, and facilitates cell adhesion to matrix components (Leask & Abraham, 2004). TGFβ1 is also an important immunoregulatory cytokine that maintains self-tolerance and T cell homeostasis, by inducing regulatory T cell differentiation and inhibiting T cell proliferation and differentiation (Bommireddy & Doetschman, 2007). TGFβ1 has a distinct biphasic nature, functioning as a potent chemoattractant for macrophages/ monocytes, neutrophils and lymphocytes during the early recruitment or activation phase of the immune response, then later suppressing these events during the resolution of an immune response (Prud'homme & Piccirillo, 2000). Recently it has been shown that TGFβ plays a dual role in autoimmune-mediated organ damage in a mouse model of lupus, where decreased TGFβ in immune cells predisposes to autoantibody production, which

then causes tissue inflammation that triggers production of anti-inflammatory cytokines such as TGFβ in target organs to counter inflammation, but the enhanced TGFβ in target organs then leads to dysregulated tissue repair and fibrogenesis (Saxena *et al.*, 2008).

Both TGFβ1 and β3 have been detected in ocular MMP (Elder *et al.*, 1997). All 3 isoforms of TGFβ have been shown in vitro to stimulate collagen contraction, proliferation and migration of human Tenon's capsule fibroblasts, and to induce conjunctival scarring in mouse model (Cordeiro *et al.*, 2000), however addition of the exogenous TGFβ3 isoform to cutaneous wounds reduces scar tissue formation, and TGFβ3 has been developed for human trials as an anti-scarring therapy (Occleston *et al.*, 2008). Studies investigating therapeutic anti-TGFβ antibodies have so far been disappointing. A recent clinical trial of anti-TGFβ1 in systemic sclerosis patients showed no improvement in the extent of skin involvement (Denton *et al.*, 2007). Despite initial promising results, subconjunctival injection of anti-TGFβ2 has not been shown to be successful in preventing conjunctival scarring following glaucoma filtration surgery (Khaw *et al.*, 2007); modification of the dosing regimen may have improved the outcomes. It is also possible that TGFβ1 plays a greater role in conjunctival scarring than TGFβ2.

1.9.2 CTGF (connective tissue growth factor)

Connective tissue growth factor (CTGF), an important downstream mediator of TGFβ-induced collagen synthesis, has been shown to be expressed at increased levels in the stroma and by conjunctival fibroblasts in ocular MMP (Razzaque *et al.*, 2003c). TGFβ blockade reduces the expression of CTGF and type I collagen by conjunctival fibroblasts (Razzaque *et al.*, 2003c). CTGF is the key profibrotic mediator of TGFβ1-induced matrix synthesis, myofibroblast differentiation and matrix contraction that is involved specifically in the TGFβ-induced tissue response to injury and not the other TGFβ-induced immune suppressor functions. It may thus be a more attractive, selective target for inhibition than TGFβ (Garrett *et al.*, 2004).

1.9.3 PDGF and FGF

Platelet derived growth factor (PDGF) is produced by activated macrophages, fibroblasts, endothelial cells and smooth muscle cells and stimulates both fibroblast proliferation and connective tissue production. Fibroblast growth factor (FGF), also produced by activated macrophages, appears to be less fibrogenic than PDGF, given that it stimulates fibroblast proliferation but not connective tissue production (Elder, 1997c).

1.9.4 Interleukin- 4 (IL-4)

Although Bernauer did not detect expression of the Th2 cytokine IL-4 in MMP, others have subsequently detected both protein and mRNA expression of IL-4 and IL-5 in MMP (Caproni *et al.*, 2003; Razzaque *et al.*, 2003a) (see *section 1.5.2* above).

Although the extent to which IL-4 participates in the progression of fibrosis can vary according to the disease, some studies indicate that IL-4 is nearly twice as efficient at mediating fibrosis as TGFβ (Wynn, 2004). IL-4 is produced by Th2 lymphocytes, mast cells, fibroblasts and eosinophils. It acts on T lymphocytes, fibroblasts and B cells. Its actions on fibroblasts include stimulating chemotaxis (Postlethwaite & Seyer, 1990), proliferation and synthesis of extracellular matrix proteins (Postlethwaite *et al.*, 1992), and prevention of apoptosis (Fujitsu *et al.*, 2005). It is a potent inducer of TGFβ and macrophage colony-stimulating factor (m-CSF) production by pulmonary fibroblasts (Jakubzick *et al.*, 2004). It also has several actions on fibroblasts in common with IL-13 (see **section 1.11**), in part because both cytokines use the same IL-4 receptor α-chain signaling pathway. The other actions of IL-4 include inducing differentiation of CD4+ cells into Th2 cells, amplifying inflammatory responses, regulating differentiation of, and increasing IgE production. Blocking the bioactivities of IL-4 results in significant inhibition of the healing process (Jakubzick *et al.*, 2004; Ong *et al.*, 1998). Recently it has been reported that IL-4 promotes tissue inflammation by suppressing generation of

Foxp3^{+} regulatory T cells, and that furthermore the combination of TGFβ and IL-4 (as found in ocular MMP) induces the generation of a distinct population of Foxp3^{-} IL-9^{+} IL-10^{+} T cells that also promote chronic inflammation (Dardalhon *et al.*, 2008).

IL-4 is produced at increased levels by ocular MMP fibroblasts compared to normal fibroblasts (Razzaque *et al.*, 2003a). IL-4 has also been detected in lymphocytes, plasma cells, mast cells and eosinophils in oral and ocular MMP (Caproni *et al.*, 1997; Caproni *et al.*, 2003). Serum levels of IL-4 are not elevated, indicating that only local tissue production of IL-4 takes place (Razzaque *et al.*, 2003a). IL-4-stimulated conjunctival fibroblasts produce increased levels of heat shock protein 47 (HSP47), type I collagen and m-CSF (Razzaque *et al.*, 2003a).

1.9.5 IL-5

IL-5 is a Th2 type fibrogenic cytokine produced by Th2 lymphocytes, mast cells, eosinophils and monocytes. It stimulates the proliferation and activation of eosinophils, which have been shown to be key cellular sources of profibrotic mediators in pulmonary fibrosis. Elevated serum levels of IL-5, as well as elevated numbers of eosinophils in the peripheral blood and conjunctival stroma, have been reported in active ocular MMP (Letko *et al.*, 2002), however the numbers of eosinophils present in comparison to other inflammatory cells is not high (6 eosinophil cells/cm^2 compared to 114 neutrophils/mm^2 or 74 T lymphocytes /mm^2) (Bernauer *et al.*, 1993b), so the role of IL-5 in fibrosis in ocular MMP is probably limited.

1.10 Summary of the fibrogenic cellular response in ocular MMP

The key cells involved in fibrosis in ocular MMP include macrophages, T cells and fibroblasts. It is possible that epithelial cells and epithelial-mesenchymal transition (EMT) also play a role in fibrotic remodeling in MMP, given that chronic mucosal injury is involved, but to the author's knowledge this has not yet been investigated in MMP.

1.10.1 Macrophages

Macrophages are essential in wound healing and promote the transition from inflammation to new tissue formation by secreting growth factors like TGFβ, IL-4 and PDGF (Mosser & Edwards, 2008). Razzaque et al have shown that local macrophage accumulation in ocular MMP is due to increased macrophage migration inhibitory factor (MIF) and macrophage- colony stimulating factor (m-CSF) expression in the epithelium and stroma. Both of these growth factors are produced by MMP conjunctival fibroblasts and their production is induced by IL-1 and TNFα and by TGFβ (Razzaque *et al.*, 2002; Razzaque *et al.*, 2004).

1.10.2 T cells

Although macrophages and fibroblasts are the main effector cells of fibrosis, the dramatic local tissue infiltration of T cells in subacute, acute and chronic ocular MMP suggests that they are likely to play a role in fibrogenesis in this disease. T cells can contribute to fibrosis both directly by elaborating cytokines which recruit and activate fibroblasts, and indirectly by activating macrophages which then release fibroblast growth factors.

Functional T cell subsets

Studies of cytokine-deficient mice have shows that fibrogenesis is strongly linked with the development of a T helper 2 (Th2) CD4+ T cell inflammatory response, involving IL-4, IL-5 and IL-13. Th2-polarized immune responses are primarily induced by disturbances at mucosal surfaces (Mosser & Edwards, 2008). In contrast, when T helper 1 (Th1) inflammatory responses dominate, and produce IFNγ, tissue fibrosis is almost completely attenuated (Wynn, 2004). The cytokine profile observed in the acute phase of MMP suggests a mixed helper T cell type 1 (Th1) and type 2 (Th2) response (see previous **section 1.7**).

T cell activation and costimulatory molecules

The process of activating T cells and the adaptive immune response first involves non-specific, reversible binding between antigen presenting cells (APCs) and T

cells via adhesion molecules, such as ICAM (intercellular adhesion molecule) interacting with lymphocyte functional antigen-1 (LFA-1). Optimal T cell activation subsequently requires two signals to produce maximal amounts of cytokines and to proliferate: T cell receptor occupancy by the antigenic peptide bound to class II major histocompatibility complex (MHC) such as HLA-DR, as well as a second nonantigen-specific “costimulatory” signal provided by T cell-APC contact. Engagement of the T cell receptor in the absence of a costimulatory signal can induce anergy (Corrigall *et al.*, 2000). Ligand-receptor pairs which play a prominent role in costimulating T cell activities include B7/CD28 which mediates afferent signals to T cells, and CD40/CD40 ligand which mediates efferent signals to the targets of T cells (Crow, 2006).

The B7/CD28 costimulatory pathway consists of two ligands on APCs, B7-1 (CD80) and B7-2 (CD86), each of which can engage CD28, the costimulatory receptor on T cells. There is also an inhibitory receptor for the B7 ligands, CTLA-4 (cytotoxic T lymphocyte- associated antigen 4 / CD152), which is upregulated after T cell activation has peaked, and limits the degree of activation. Both B7-1 (CD80) and B7-2 (CD86) are constitutively expressed on dendritic cells, and their expression on macrophages and B cells and other APCs can be upregulated by activation with IFNγ or lipopolysaccharide (Bhatia *et al.*, 2006). Whilst B7-1 (CD80) is expressed in a tightly regulated manner on activated but not resting B cells, B7-2 (CD86) is constitutively expressed on the cell surface of APCs and rapidly upregulated upon interaction with CD28, with peak expression at 48 hours. Expression of B7-1 (CD80) is slowly induced and is stable for 4-5 days. CD80 and CD86 have been shown to modulate Th1/Th2 T cell differentiation. CD80 preferentially favours Th1-type T cell differentiation, while CD86 augments IL-4 production and Th2 type T cell responses (Kuchroo *et al.*, 1995).

The CD40/CD40 ligand pathway plays a key role in T cell effector functions that determine the overall activity of the immune response (Crow, 2006). CD40 is

expressed on all APCs, including B cells and endothelial cells. CD40 ligand (CD40L or CD154) is expressed on activated $CD4^+$T cells and on a subset of $CD8^+$T cells and natural killer cells. CD40 stimulation triggers important signals for antibody production by B cells, and strongly induces B7 and MHC expression on APCs, thus increasing antigen presentation by an important co-stimulatory pathway. The role of CD40 signalling in regulation of inflammation and fibrosis has been demonstrated in the lung (Kaufman *et al.*, 2001; Sempowski *et al.*, 1997). During an inflammatory response, fibroblasts increase their expression of CD40, and interactions between tissue fibroblasts and infiltrating T lymphocytes via the CD40/CD40L pathway have been proposed to to powerfully costimulate T lymphocyte proliferation as well as induce fibroblasts to produce proinflammatory and chemoattractant cytokines. This in turn may lead to fibroblast proliferation and extracellular matrix synthesis. Chronic stimulation through CD40 may hence lead to fibrosis. Furthermore, it has been reported that primary human lung fibroblasts express CD40 ligand (CD154), which is usually not known to be expressed on non-bone marrow-derived structural cells (Kaufman *et al.*, 2004). This expression is increased in fibroblasts from scarred lungs, and may promote fibroblast activation and interaction with CD40-expressing cells.

In ocular MMP, Tesavibul et al have found upregulated subepithelial stromal CD86 but not CD80 expression in active disease, which is consistent with the development of a Th2 type fibrogenic response (Tesavibul *et al.*, 1998). There do not appear to be any studies investigating the role of CD40 in MMP.

Cell surface expression of ICAM, HLA-DR, CD80, CD86 or CD40 by fibroblasts acting as APCs in ocular MMP could indicate a mechanism for cross-talk between fibroblasts and T cells, which thus promotes fibroblast and T cell activation, chronic inflammation and fibrosis.

Direct lymphocyte-fibroblast interactions in wound healing and fibrosis

Apart from the indirect effect of the Th2 cell-secreted cytokines IL-13 and IL-4 acting on fibroblasts to cause fibrosis, there is evidence to suggest that direct interactions between T lymphocytes and fibroblasts perpetuate chronic inflammation, and this may hence lead to further fibrosis and remodelling. In rheumatoid arthritis, fibroblast-like synoviocytes and T cells in co-cultures have been shown to interact in a similar manner to professional APC-T cell interactions, to induce a proliferative response and increased release of inflammatory mediators (Tran *et al.*, 2008). Ligation of CD40-positive human intestinal fibroblasts by CD40 ligand-positive T cells generates fibroblast-derived chemoattractants that induce T cell migration (Vogel *et al.*, 2004). T cell viability is significantly greater when T cells are co-cultured with human Tenon's fibroblasts, and fibroblast production of IFNβ, which prevents T cell apoptosis, appears to be the mechanism by which this occurs (Chang *et al.*, 2001).

1.10.3 Fibroblasts

Activation and proliferation of fibroblasts is primarily responsible for the scarring response, and this has been detected in MMP (Bernauer *et al.*, 1993a; Caproni *et al.*, 1997). Significant staining for both ICAM-1 (intercellular adhesion molecule-1) and VCAM (vascular cell adhesion molecule) on perivascular fibroblasts, indicating fibroblast activation, has been detected in oral and cutaneous tissue affected by MMP (Giomi *et al.*, 2005).

The absence of detectable fibrogenic cytokines TGFβ, PDGF and FGF in chronic ocular MMP, has led to a hypothesis that in chronic disease the fibroblasts may be hyperactivated and behave independently from cytokine influences, to sustain their own growth and cause fibrosis. There have been limited studies investigating this hypothesis. Ocular MMP conjunctival fibroblasts are reported to be hyperproliferative in culture compared with normal conjunctival fibroblasts (Roat *et al.*, 1989). Preliminary studies have also described expression of the proto-

oncogene *c-myc* by ocular MMP fibroblasts (Hunt LE, et al. IOVS 1991;32:ARVO Abstract 938), and ultrastructural changes indicating increased protein synthesis which persist in culture (Biesman BS et al. IOVS 1994;Suppl 35(4):ARVO Abstract 170). Increased expression of collagen type I, TGFβ1, macrophage migration inhibitory factor, macrophage-colony stimulating factor, connective tissue growth factor and heat shock protein 47 by ocular MMP conjunctival fibroblasts compared with normal conjunctival fibroblasts has also been reported (Razzaque *et al.*, 2003b; Razzaque *et al.*, 2004).

Fibroblast synthesis of ECM (extracellular matrix) leads to formation of new connective tissue in the substantia propria. The degree of scarring is dependent on the balance between collagen synthesis by fibroblasts and collagen degradation controlled by matrix metalloproteinases (mmps) and their inhibitors (tissue inhibitors of matrix metalloproteinases, TIMPs). Unpublished observations suggest that expression of mmp-1 and -14 and TIMP-1,-2, and -3 are increased in fibroblasts isolated from the conjunctiva of ocular MMP patients (Razzaque *et al.*, 2001).

The substantia propria of ocular MMP conjunctiva shows increased type I and type III collagen staining (Dutt *et al.*, 1996; Razzaque *et al.*, 2003b). These collagen fibrils are highly disorganized (Galbavy & Foster, 1985). There is also aberrant type IV and type VII collagen production, which appears to be repair in response to the basement membrane zone damage. Formation of collagen in ocular MMP appears to involve heat shock protein 47 (HSP47), a collagen-binding protein which plays an important role in the biosynthesis of procollagens. Razzaque et al (Razzaque *et al.*, 2003b) have shown that HSP47 is expressed at increased levels by stromal conjunctival fibroblasts in ocular MMP, and its production is increased by TGFβ1.

Fibroblast functions in wound healing and fibrosis

The fibroblast is the central cell in wound healing and fibrosis. The functions of fibroblasts have been arbitrarily subdivided into four aspects: proliferation, migration, matrix synthesis and remodelling. Myofibroblasts, which are specialized differentiated fibroblasts, are involved in the latter two aspects of extracellular matrix synthesis, and tissue contraction and remodelling (Tomasek *et al.*, 2002). In addition, fibroblasts function as regulators of the inflammatory response, prevent T cell apoptosis and contribute to persistent inflammation (Flavell *et al.*, 2008). Fibroblast-induced prevention of T cell apoptosis could be an explanation for the persistent T cell infiltrate which has been observed in chronic ("white inflammation") ocular MMP (Bernauer *et al.*, 1993b; Elder, 1997c; Sacks *et al.*, 1989).

In addition to being derived from resident mesenchymal cells, fibroblasts and myofibroblasts can be derived from other sources, including epithelial cells via epithelial-mesenchymal transition (EMT), and from circulating bone marrow-derived cells called fibrocytes (Wynn, 2008). This may in part account for fibroblast heterogeneity that exists both within and between tissues. Fibroblasts from distinct bodily sites exhibit differences in their proliferative and biosynthetic capacity (Flavell *et al.*, 2008), and this finding has subsequently been found to be true of fibroblasts from the same connective tissue bed, which have been isolated from different morphological locations within that connective tissue bed (Abraham *et al.*, 2007). Thus, it is believed that fibroblasts exist in distinct subpopulations within connective tissues, that can be considered distinct differentiated cell types.

Migration

Fibroblast migration plays an important role in fibrosis. This is controlled by specific polypeptides acting as chemoattractants. Overproduction of chemotactic factors may result in excessive scarring. Other factors influencing fibroblast motility include mechanisms to clear space within the extracellular matrix, which

enable fibroblasts to penetrate and move through the matrix. Space-clearing can be achieved by phagocytosis, as well as by enzymes such as plasmin, plasminogen activator, and matrix metalloproteinases (mmps) which include collagenases (mmp-1, 8, 13), gelatinases (mmp-2, 9) and stromelysins (mmp-3, 10 and 13). The movement of the cells may, in itself, cause traction and wound contraction.

Extracellular matrix synthesis, remodelling and matrix contraction

Fibroblasts are the principle biosynthetic cells secreting the extracellular matrix (ECM) components fibronectin, glycosaminoglycans and tropocollagen, which then cross-link to form collagen. Type III collagen is the first type of collagen to fill a wound. Type III is replaced by type I collagen, the mature form, with time. Collagen and ECM production by fibroblasts is remodelled with continuous synthesis and breakdown of the matrix. The amount of ECM turnover at the wound depends on the extent of matrix metalloproteinase activity (Wong *et al.*, 2002). Apart from the key role of myofibroblasts in matrix contraction (Tomasek *et al.*, 2002), fibroblast-mediated matrix contraction *in vitro* has also been shown to involve matrix metalloproteinase (mmp) production, and mmp inhibition reduces this matrix contraction, as well as reducing collagen synthesis (Daniels *et al.*, 2003a). Matrix metalloproteinase physiology is complex and incompletely understood, given that increased mmp production can either increase scar tissue formation by promoting fibroblast migration and wound contraction, or decrease scar tissue by favouring matrix breakdown and reducing the net level of collagen present.

<u>Fibroblast-mediated chronic inflammation</u>

Although fibroblasts were originally thought of as merely structural cells, it is now clear that they take a much more active role in defining a tissue's microenvironment by synthesizing chemokines, cytokines and growth factors (Filer *et al.*, 2006), and provide navigation cues for retention of leukocytes within tissues via a stromal address code created by the expression of crucial molecules

(Parsonage *et al.*, 2005). Fibroblasts can also participate in direct cross-talk with lymphocytes by expressing CD40 (Kaufman *et al.*, 2001).

It has been proposed that fibroblasts regulate the switch from acute resolving to chronic persistent inflammation (Buckley *et al.*, 2001). Failure to switch off the inflammatory stimulus from fibroblasts may lead to the inappropriate survival and retention of leukocytes within inflamed tissue. Production of interferon-β by human Tenon's fibroblasts, as a mechanism by which fibroblasts prevent T cell apoptosis and perpetuate chronic conjunctival inflammation, has been reported (Chang *et al.*, 2002). Dysregulated expression of CD40 ligand (CD154), which is usually only expressed by bone marrow-derived non-structural cells, has been reported in fibrotic lung tissue fibroblasts. Selective expansion of fibroblasts expressing such CD40 ligand could result in chronic autocrine and paracrine activation of fibroblasts via this pathway (Kaufman *et al.*, 2004).

Fibroblasts derived from diseased fibrotic tissue display an autonomously activated phenotype

Whilst inflammation typically precedes the development of fibrosis in most fibrotic disorders, some experimental models suggest that fibrosis is not always characterized by persistent inflammation, and that to a degree, the mechanisms regulating fibrosis are distinct from those controlling inflammation (Stramer *et al.*, 2007). In line with this viewpoint, fibroblasts derived from patients with systemic sclerosis, pulmonary fibrosis, colonic fibrosis and renal fibrosis have been shown to display a constitutively activated phenotype in the absence of the fibrotic tissue milieu, with aberrations in processes that govern nearly every aspect of the fibroproliferative response (proliferation, resistance to apoptosis, motility, contractile function, matrix synthesis) (Ramos *et al.*, 2001; Rieder *et al.*, 2007; Schuttert *et al.*, 2003; Varga & Abraham, 2007; Wallach-Dayan *et al.*, 2007). Whilst the abnormal phenotype has initially emerged in the context of exogenous signals from matrix, cytokines, chemokines and growth factors, it is retained during many

passages *in vitro*, indicating fundamental changes in gene expression pathways (Larsson *et al.*, 2008).

Together, this data suggests that in order to inhibit fibrosis in ocular MMP, local therapy targeting abnormally activated fibroblasts, or preventing the mechanisms leading to the abnormally activated phenotype, may be necessary. Furthermore, this may control any ongoing fibroblast-stimulated chronic inflammation.

1.11 Rationale for selecting TNFα for investigation

Tumour necrosis factor-alpha (TNFα) is an important proinflammatory cytokine produced by macrophages, T and B lymphocytes, natural killer cells, neutrophils, smooth muscle cells, endothelial cells, and fibroblasts. TNFα exists in 2 forms, a membrane-bound form and a soluble form, both of which are functional and contribute to the effects of TNFα. TNFα has multiple actions, including 1) activating macrophages, neutrophils and cytotoxic T cells, 2) upregulating endothelial cell adhesion molecules to enable leukocyte entry into tissues, 3) being a comitogen for T and B cells and 4) inducing IL-2 receptor on activated T cells, and 5) having effects on fibroblasts.

The effects of TNFα on fibroblasts and fibrosis are controversial because whilst some studies show anti-fibrotic effects of TNFα *in vitro* on dermal fibroblasts (Chizzolini *et al.*, 2003; Goldberg *et al.*, 2007) and on corneal fibroblasts (Saika, 2007), others show profibrotic effects on fibroblasts *in vitro* (Chou *et al.*, 1996; Sullivan *et al.*, 2005; Theiss *et al.*, 2005). Moreover, i*n vivo* animal studies of TNFα antagonists and mice deficient in TNF receptors indicate that inhibition of TNFα signaling can prevent fibrosis (Farivar *et al.*, 2005; Guo *et al.*, 2001; Liu *et al.*, 1998; Piguet & Vesin, 1994; Sudo *et al.*, 2005). It has been proposed that these differences in results might be explained by TNFα playing a major proinflammatory role in triggering fibrosis in animal models, and the direct *in vitro* anti-fibrotic effects of TNFα on fibroblasts might be outweighed in animal models of fibrosis by its

important role in driving inflammation (Distler *et al.*, 2008). Furthermore, a recent report suggests that in clinical practice (rather than in experimental models), TNFα antagonists are beneficial in the treatment of fibrotic disorders such as systemic sclerosis (Lam *et al.*, 2007).

TNFα antagonist therapy has been shown to safely and successfully control inflammation in autoimmune diseases such as rheumatoid arthritis and uveitis (Furst *et al.*, 2008; Hale & Lightman, 2006). Apart from TNFα inhibition, this therapy has also been shown to induce regulatory T cell activity in rheumatoid arthritis (Ehrenstein *et al.*, 2004), and to induce T cell apoptosis in Crohn's disease (van den Brande *et al.*, 2003). It would be opportune to use this therapy to control inflammation in MMP, and case reports have described seven MMP patients who improved following TNFα antagonist therapy (see **section 1.4.4**), however there are limited studies investigating whether TNFα is expressed in MMP tissues (see **section 1.7.1**). Tissue expression appears to be important because synovial expression of TNFα appears to be a significant predictor of response to TNFα antagonists in rheumatoid arthritis (Wijbrandts *et al.*, 2007).

Furthermore, given that fibroblasts isolated from different anatomical sites exhibit different functional properties (Flavell *et al.*, 2008), the effects of TNFα on fibroblasts derived from the conjunctiva needs to be established before conducting larger controlled studies of the efficacy of TNFα antagonists in MMP, given that the therapy could potentially be profibrotic and could therefore worsen scarring. Leonardi et al have reported that TNFα increased mmp-9 and also mmp-1 production, and decreased timp-1 production in response to TNFα stimulation, by conjunctival fibroblasts derived from vernal keratoconjunctivitis (VKC) patients (Leonardi *et al.*, 2003). The phenotype of VKC fibroblasts may be different from that of normal conjunctival fibroblasts, so the findings reported by Leonardi cannot be extrapolated to normal conjunctival fibroblasts, nor to ocular MMP conjunctival fibroblasts. Leonardi has reported elsewhere that normal conjunctival fibroblasts

secrete increased amounts of chemokines (IL-6, IL-8, MCP-1, RANTES, IP-10) in response to TNFα stimulation, thus confirming that normal conjunctival fibroblasts have receptors for and respond to TNFα stimulation (Leonardi *et al.*, 2006).

Therefore, one of the hypotheses investigated in my study was that TNFα is involved in inflammation in ocular MMP, and may have a profibrotic effect on conjunctival fibroblasts.

1.12 Rationale for selecting IL-13 for investigation

IL-13 is a major pro-fibrotic mediator which is produced by Th2 helper T cells, mast cells and basophils. It regulates inflammation, mucus production, tissue remodeling and fibrosis in the lung, as well as playing a critical role in the response to nematode and intracellular parasite infections (Wynn, 2003). It has become an important therapeutic target for a number of chronic inflammatory-fibrotic diseases including idiopathic pulmonary fibrosis, liver fibrosis and asthma, because animal models where IL-13 has been inhibited independently of IL-4 have identified IL-13 as the dominant effector cytokine of fibrosis (Blease *et al.*, 2001; Chiaramonte *et al.*, 1999; Kumar *et al.*, 2002). IL-13 appears to exert its profibrotic activities via inducing production of latent TGFβ in macrophages, and stimulating enzymes that cleave and activate latent TGFβ (Wynn, 2008). It also appears to trigger fibrosis via TGFβ-independent mechanisms (Kaviratne *et al.*, 2004).

T cells are prominent in the conjunctival substantia propria in ocular MMP (Bernauer *et al.*, 1993b), and whilst expression of the Th2 cytokines IL-4 and IL-5 has been detected (Caproni *et al.*, 2003; Razzaque *et al.*, 2003a), it is not known whether IL-13 is expressed in ocular MMP. Anti-TGFβ therapies have not been as successful at preventing conjunctival scarring as initially hoped (Khaw *et al.*, 2007), and it is possible that, if IL-13 is detected to be present in the chronic inflammatory-fibrotic setting of ocular MMP, inhibition of IL-13 could block both TGFβ-mediated and non-TGFβ-mediated mechanisms of fibrosis.

IL-13 shares many functional activities with IL-4 because both cytokines bind to the type 2 IL-4 receptor (also known as IL-13Rα1, composed of heterodimeric IL-4Rα and IL-13Rα1 subunits), which is expressed on B cells, NK cells, monocytes, mast cells, endothelial cells and fibroblasts. In addition, IL-13, but not IL-4, binds with 100-fold higher affinity for another receptor chain, IL-13Rα2, than IL-13Rα1. The IL-13Rα2 chain was formerly considered to only function as a decoy receptor, but more recently it has been shown in macrophages that IL-13 signaling via induction of cell-surface expression of IL-13Rα2, activates the TGF-β1 promoter to synthesise TGF-β and cause fibrosis (Fichtner-Feigl *et al.*, 2006). Induction of cell-surface IL-13 Rα2 requires the presence of both IL-13 (or IL-4) and TNFα. My studies of both TNFα and IL-13 in ocular MMP could therefore be relevant to investigating whether this pathway is involved in fibrosis in ocular MMP.

IL-13 is unique in that it is not thought to exert any control over T cell function (Hershey, 2003), so unlike IL-4 it does not appear to be important in the initial differentiation of CD4+T cells into Th2-type cells, but rather appears to be important in the effector phase of inflammation and repair, via its effects on monocytes/ macrophages, dendritic cells, fibroblasts and other inflammatory and stromal cells. Somewhat paradoxically, IL-13 was originally described as a T cell derived cytokine that inhibits inflammatory cytokine production, but its other effector functions were discovered from several unique animal models which promoted or blocked IL-13 activity (Wynn, 2003). It has been proposed that the greater fibrogenic activity of IL-13 over IL-4 may in part be related to the much higher concentrations of IL-13 present, which often exceeds those of IL-4 by a factor of 10-100, and partly also due to IL-13 using an additional fibrogenic signaling pathway, which is distinct from the IL-4Rα pathway which it has in common with IL-4 (Wynn, 2008).

IL-13 has been shown to exert direct pro-fibrotic effects on fibroblasts isolated from human lung and skin (Ingram *et al.*, 2003; Kohyama *et al.*, 2004; Liu *et al.*, 2002; Saito *et al.*, 2003) including stimulation of proliferation, chemotaxis and collagen gel contraction, but fibroblasts isolated from different bodily sites exhibit different functional properties and site-specific gene expression (Flavell *et al.*, 2008), so these effects cannot be extrapolated to fibroblasts from other tissues such as the conjunctiva. The effects of IL-13 on stimulating normal human conjunctival fibroblast proliferation, preventing apoptosis and decreasing matrix metalloproteinase 3 synthesis have been reported (Fujitsu *et al.*, 2005; Fukuda *et al.*, 2006), but its effects on other functional activities carried out by normal conjunctival fibroblasts have not been fully investigated. Leonardi et al have reported that IL-13 increased collagen synthesis, decreased production of matrix metalloproteinase 1 and increased production of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) by conjunctival fibroblasts derived from vernal keratoconjunctivitis (VKC) patients (Leonardi *et al.*, 2003), which may have a different phenotype to that of normal conjunctival fibroblasts, and ocular MMP conjunctival fibroblasts. Normal conjunctival fibroblasts do appear to have receptors for, and respond to IL-13, given that increased secretion of eotaxin-1 and MIG has been reported following IL-13 stimulation of normal conjunctival fibroblast cultures (Leonardi *et al.*, 2006).

To our knowledge there are no previous studies evaluating the role of IL-13 in ocular or extraocular MMP. Given the prominence of T cells in the substantia propria in ocular MMP, we were also interested in potential fibroblast-T cell interactions and their contribution to conjunctival fibrosis. We therefore chose to investigate the hypothesis that IL-13 and fibroblast-T cell interactions play an important role in fibrosis in ocular MMP.

1.13 Conclusion

Immunosuppressive therapy is standard treatment for controlling inflammation in ocular MMP, but how well it controls fibrosis is unknown. It has been reported that systemic corticosteroids can prevent fibrosis associated with acutely inflamed disease. When inflammation has been resistant to conventional immunosuppressive therapy, a handful of reports have described improvement following systemic TNFα antagonist treatment in ocular MMP, but the scientific rationale for such treatment is weak as there are limited studies demonstrating tissue expression of TNFα in MMP. TNFα appears to have either antifibrotic or profibrotic effects depending on the fibroblast type and experimental setting, so investigating its effects on conjunctival fibroblasts is important when considering use of TNFα antagonist treatment in ocular MMP.

T cells are prominent in the substantia propria during acute, subacute and chronic ocular MMP, and are likely to play a key role in fibrosis. The type 2 helper T cell-derived cytokine IL-13 is a key profibrotic mediator acting upstream of TGFβ in other chronic inflammatory fibrotic diseases of the liver and lung, and whether IL-13 is expressed in ocular MMP, and its effects on conjunctival fibroblasts, are unknown. Finally, there is some evidence that conjunctival fibroblasts derived from patients with ocular MMP maintain a hyperproliferative phenotype and show increased secretion of inflammatory cytokines, but these findings have not been investigated further.

1.14 Aim and Hypotheses

The aim of this thesis was to investigate potential candidate molecules and mechanisms involved in conjunctival fibrosis in ocular MMP, for the purpose of proposing future adjuvant systemic and/or local anti-fibrotic therapies, to be used in conjunction with systemic immunosuppressive therapy.

My overall hypothesis is that it is inflammation, rather than acquired characteristics of the fibroblasts, which drives fibrosis in ocular MMP.

My studies were designed to investigate the following hypotheses:

1. That current immunosuppressive treatment in ocular MMP controls inflammation but not necessarily fibrosis
2. That more rapid control of inflammation in ocular MMP using pulse intravenous methylprednisolone may more effectively prevent fibrosis
3. That the acute inflammatory cytokine TNFα is involved in inflammation in ocular MMP, and may have a profibrotic effect on conjunctival fibroblasts
4. That the type 2 helper T cell cytokine IL-13, and fibroblast-T cell interactions play an important role in fibrosis in ocular MMP
5. That conjunctival fibroblasts derived from ocular MMP patients behave in a functionally similar manner to normal conjunctival fibroblasts, and are not autonomously profibrotic

Chapter 2

Retrospective review of current immunosuppressive therapy in ocular mucous membrane pemphigoid

2.1 Introduction

The goals of treatment of ocular mucous membrane pemphigoid (MMP) with immunosuppressive therapy are the suppression of inflammation and arrest of cicatrisation, whilst minimising drug toxicity and potential long term adverse effects including neoplasia.

Although several studies have reported the response of ocular MMP to individual therapeutic agents such as, for example, cyclophosphamide and high dose prednisolone (Elder *et al.*, 1995; Foster, 1986), dapsone (Foster, 1986), methotrexate (McCluskey *et al.*, 2004) or sulfapyridine (Elder *et al.*, 1996b), in practice such treatments may only be received for a certain time period before they need to be switched to alternative agents because of side effects or lack of control of inflammation. Combination therapy may also be necessary. Treatment guidelines based on using dapsone as initial therapy for mild to moderate inflammation and cyclophosphamide as initial therapy for severely active inflammation, azathioprine for patients intolerant of dapsone, and subsequent substitutions or additions of agents depending on disease activity and drug tolerance have previously been described (Foster, 1986; Tauber *et al.*, 1991).

Only a few studies have investigated the overall results of such sequential conventional immunosuppressive therapy regimens in terms of success in controlling inflammation, and success in arresting cicatrisation (Elder *et al.*, 1996a; Miserocchi *et al.*, 2002; Tauber *et al.*, 1991).

2.2. Aim

The aim of the study in this chapter was to establish, in a large cohort of ocular MMP patients, how well a stepladder treatment strategy using conventional immunosuppressive therapy controls both inflammation and cicatrisation in ocular MMP.

2.3 Specific methods and research design

Given the rare nature of the disease and the requirement for prolonged follow up, a retrospective case note review was the most suitable method for investigating treatment outcomes in a large number of patients. The clinical records of 115 consecutive patients with ocular MMP attending Moorfields Eye Hospital (London, United Kingdom) from January 1994 to August 2005 were reviewed.

2.3.1 Inclusion criteria

The inclusion criteria were patients with a clinical diagnosis of ocular MMP and at least 6 months follow-up at Moorfields, after commencing systemic immunosuppression. A clinical diagnosis of ocular MMP was made if there was progressive conjunctival cicatrisation, after excluding other causes of progressive scarring such as Sjögren's syndrome, atopic keratoconjunctivitis and rosacea (Thorne *et al.*, 2004) by history, examination and investigations. Drug-induced ocular MMP was diagnosed in patients with clinical features of MMP who had a history of causative medication use and progression of clinical signs despite ceasing the causative therapy.

All reasonable attempts were made to obtain a positive immunopathological tissue diagnosis by conjunctival and/or other mucosal or skin biopsies; however, a negative or inconclusive biopsy did not exclude the diagnosis of ocular MMP (Ahmed *et al.*, 2004; Bernauer *et al.*, 1994), in the presence of characteristic clinical features.

2.3.2 Data collected

I collected the following data from the clinical records: date of diagnosis, biopsy results, extraocular manifestations, details of therapies, treatment-related side effects, and reasons for changes in therapy. The ocular findings analyzed included best-corrected visual acuity, conjunctival inflammation and the most advanced Mondino and Foster stage (Foster, 1986; Mondino & Brown, 1981), at the first visit

and at last follow-up. It was not possible to record the Tauber (modified Foster) stage because the number and percentage extent of symblephara had not been consistently recorded in the notes. Conjunctival inflammation was graded as 0 (absent) to 4 (severe) (Elder *et al.*, 1995; Foster, 1986). The worst eye of each patient was selected according to the following criteria: highest Mondino stage, worst inflammation, worst visual acuity, or the right eye if both eyes were equal.

2.3.3 Immunosuppressive treatment regimen: stepladder algorithm

Immunosuppressive regimens employed either 'step-up' or 'step-down' treatment depending on the activity of MMP, as previously described (Rauz *et al.*, 2005) (**Figure 2.1**). Briefly, for 'step-up' therapy, dapsone (diaminodiphenylsulfone) (25-50 mg bd) or sulphapyridine (500mg od to bd, or sulfasalazine 500-1000 mg bd when sulphapyridine was unavailable) was prescribed for mild or moderate inflammation. For disease not responding after 2-3 months of first-line therapy, azathioprine (1-2.5mg/kg/day) was added (when there was some response) or substituted (if there was no response). For those intolerant of or not responding to azathioprine, mycophenolate mofetil (500mg-1g bd) was used. Severe inflammatory disease was treated with cyclophosphamide (1-2mg/kg/day). As optimal effects with cyclophosphamide are not achieved until 8 weeks following the initiation of therapy, an adjunctive reducing regimen of oral corticosteroids (prednisolone commencing at 1 mg/kg/day) was employed, sometimes with intravenous pulses of methylprednisolone (500mg-1g, up to three doses over three days). Due to an increased risk of bladder carcinoma (Knight *et al.*, 2004), the safe duration of treatment with cyclophosphamide is limited to 12 months, so immunosuppression was "stepped-down" to the less toxic medications azathioprine, mycophenolate mofetil, methotrexate or dapsone at the end of this period. Combination therapy was used for resistant cases, and included the combination of a sulpha (dapsone, sulphapyridine) and myelosuppressive agent (azathioprine, mycophenolate, cyclophosphamide), and/or the addition of prednisolone (either as a maintenance dose of ≤7.5mg/day or as a brief tapering 6-

8 week course). Blood pressure, weight, urinalysis, and blood tests were evaluated regularly to screen for drug-related side effects records.

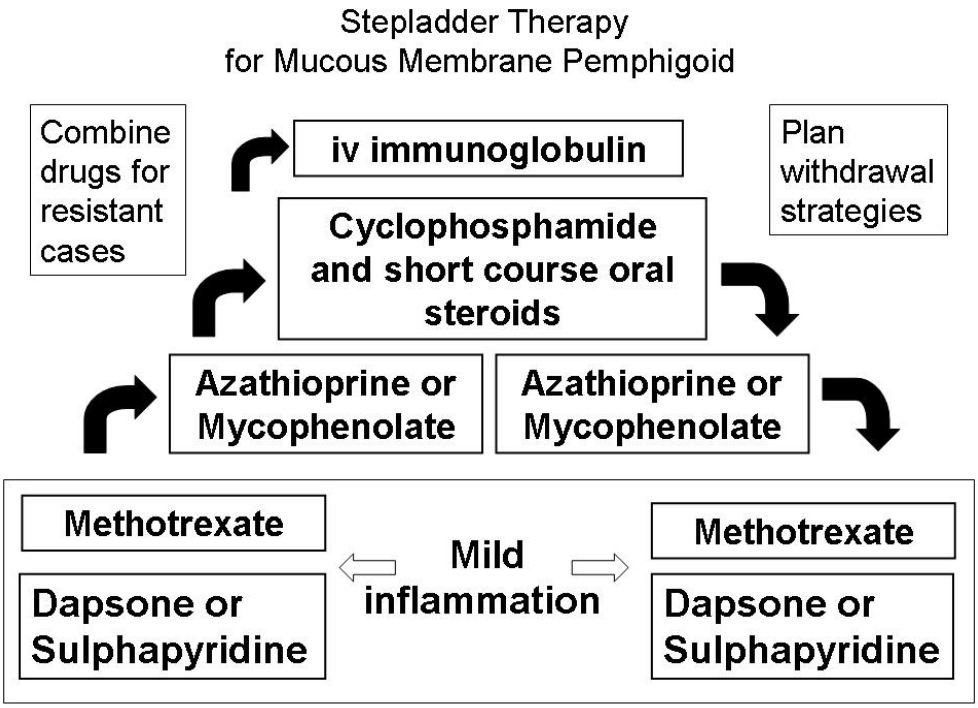


Figure 2.1 Algorithm showing stepladder immunosuppression strategy

For severe disease, commence with cyclophosphamide and plan introduction of less toxic drugs, and withdrawal of cyclophosphamide once the disease is under control. For mild disease use dapsone (or sulfapyridine if dapsone is not tolerated) and step-up to azathioprine or mycophenolate. For treatment failures with this regimen, progress to cyclophosphamide. Oral prednisolone for 6 weeks is usually combined with cyclophosphamide, whilst awaiting the commencement of immunosuppressive effect. Combinations of a sulpha-based agent (dapsone or sulphapyridine) with a myelosuppressive agent (cyclophosphamide, azathioprine, mycophenolate) and prednisolone are also effective. iv = intravenous.

2.3.4 Treatment episode and outcome definitions

A **treatment episode** was defined by the use of a single immunosuppressive agent or therapeutic combination of agents. For each treatment episode, the success of immunosuppressive therapy in controlling inflammation was classified as a success (S), qualified success (QS) or failure (F). **"Success" (S)** was defined as where there was induction of quiescence with a quiet, white eye for at least 3 months from the commencement of therapy. If, following initial success, a change in therapy was necessary due to a reactivation of inflammation uncontrolled by the current regimen, this was defined as **"success then inflammation" (Si)**; if a change in therapy, following initial success, was necessary due to adverse effects, this was defined as **"success then adverse effects" (Sa)**. **"Qualified success"(QS)** was defined as where inflammation was partially controlled, with some residual inflammation. **"Failure" (F)** was defined as where there was either **no response and persistent inflammation (Fi)**, or where therapy had to be withdrawn within 3 months, due to **unacceptable side effects, before a response could be expected (Fa)**.

Treatment episodes were defined according to the principal agent used (cyclophosphamide, azathioprine, mycophenolate, sulphapyridine or sulfasalazine, dapsone), either as monotherapy or in combination therapy. When treatment was stopped after a period of quiescence, and if it was not necessary to reinstitute immunosuppression for at least 6 months, disease was described as being in remission. The induction of quiescence was a criterion for success (S), rather than the permanence of quiescence.

2.3.5 Ethics approval and statistical analysis

The study was conducted after approval from the Moorfields Eye Hospital research governance and ethics committee. Fisher's exact test was used to assess for any association between outcome after the first treatment episode and treatment. The

Wilcoxon rank sum test was used to assess for any evidence of association between progression of cicatrisation (defined by progression in Mondino stage) and each of maximum recorded level of inflammation and duration of disease. All analyses were conducting using Intercooled Stata software version 9.0 for Windows *(StataCorp LP, Texas LA)*.

2.4 Results

115 patients (223 eyes with ocular MMP) received immunosuppression. The female: male ratio was 1.25:1. The mean age at diagnosis was 66.9 years (standard deviation 14.4 years, range 17-92 years). Unilateral disease was present in 6% (7/115) of patients, of whom 1 had unilateral drug- induced pemphigoid. There were 3 patients with drug-induced pemphigoid and 2 patients with ocular MMP developing as a sequela of Stevens-Johnson syndrome. The median duration of disease was 4.25 years, with an inter-quartile range of 2.17-8.67 years. For 90% of patients the duration of disease was within 14 years, and the longest duration was 25 years (1 patient).

2.4.1 Biopsy results

Of the 62 patients in whom biopsy results were available, 69% (43/62) were positive. 2 patients had positive histology. Direct immunofluorescence (DIF) studies in the remaining 41 patients were positive. Conjunctival biopsies had been performed in 79% (49/62) of these patients, of which 61% (30/49) showed a positive DIF result. Of the 14 buccal biopsy results available, 78.6% (8/14) were positive on DIF microscopy, whilst 57.1% (4/7) of the skin biopsies were DIF positive.

2.4.2 Extraocular disease

Extraocular manifestations were present in 50% (58/115). These included lesions of the mouth in 78%, pharynx 15%, oesophagus 14%, nose 33%, skin 22%, larynx 14%, rectum 5% and genitals 9%. Associated autoimmune disease was present in 16% (18/115).

2.4.3 Treatment

Three hundred and eighty-eight treatment episodes occurred. Twelve individual agents were used in 45 different therapeutic permutations, dictated by both disease severity and medication toxicity. A successful outcome occurred in 50%

(196/388) of treatment episodes, and 27% (104/388) were considered a QS. Inflammation thus responded to immunosuppression in 77% (300/388) of the treatment episodes. Treatment failed in 23% (88/388) of episodes, of which 51% (45/88) failed due to adverse side effects (Fa), and 49% (43/87) failed due to inability to control inflammation (Fi). The predominant outcome of each patient's treatment course, calculated by determining the outcome present for the greatest duration of the treatment course, was S in 75% (86/115), QS in 20% (23/115) and F in 5% (6/115).

2.4.4 Stepladder Therapy

Of the 115 patients who received treatment, 26 patients (23%) underwent only one treatment episode, 30 patients underwent two treatment episodes (26%), and the number of treatment episodes increased to a maximum of ten episodes in three patients (3%). The reasons for further therapy are shown in **Table 2.1**. Immunosuppressive treatment was "stepped-up" in 52% (60/115) of patients, "stepped-down" in 10% (12/115) of patients, and remained at the same level on the therapeutic stepladder in 38% (43/115).

Table 2.1 Reasons for further immunosuppressive therapy

Reason for further therapy	n (%)*
Failure due to poor inflammatory control (Fi)	40 (14.7%)
Failure due to adverse side effects (Fa)	42 (15.4%)
Incomplete response (QS)	90 (33.0%)
Reactivation following initial success (Si)	25 (9.2%)
Adverse side effects following initial success (Sa)	38 (13.9%)
Maximum duration cyclophosphamide therapy	21 (7.7%)
Remission then relapse	5 (1.8%)
Patient stopped therapy then required recommencement	3 (1.0%)
Physician choice or other reason	9 (3.3%)

*Number of treatment episodes (% of treatment episodes requiring further therapy).

Fi = failure due to poor inflammatory control; Fa = failure due to adverse side effects; QS = qualified success; Si = initial success followed by reactivation; Sa = success followed by adverse side effects

2.4.5 Final Outcome

The final outcome of treatment was a white quiet eye in 70% (81/115) of patients and 78.5% (175/223) of eyes. There was residual minimal or mild inflammation in 21.5% (48/223) of eyes and 30% (34/115) of patients. Of the 48 eyes with residual inflammation, 26 (54%) continued to receive treatment, treatment had ceased in 16 (33%) eyes, and 6 eyes (3 patients) were lost to follow-up. In the 16 residually inflamed eyes where treatment had ceased, side effects prevented further treatment in 5 eyes (3 patients), other illness unrelated to therapy resulted in treatment cessation in 2 eyes (1 patient), the residual inflammation was minimal and fluctuating and the risks of treatment were felt to outweigh the benefits in 6 eyes (4 patients), and the patient wished to stop treatment in 3 eyes (3 patients). By the end of the study, 43.5% (50/115) of patients had ceased treatment. Of these 50 patients, both eyes were white in 70% (35/50) and one or both eyes were residually inflamed in 30% (15/50). Of the 65 patients (56.5%) in whom treatment was continuing, both eyes were white in 71% (46/65) and one or both eyes were residually inflamed in 29% (19/65). Remission off treatment for at least 6 months occurred in 16 patients (14%). Four additional patients suffered relapses following periods of remission off treatment lasting 4 to 24 months. Thus the proportion in remission was 17% [(16 + 4)/115] and relapse occurred in 20% (4/20).

2.4.6 Side effects

The adverse effects of therapy are listed in **Table 2.2**. Three patients suffered major adverse effects. Two patients on cyclophosphamide developed pancytopenia requiring transfusion. A further patient developed severe anaemia during sulphapyridine treatment; therapy was later changed to cyclophosphamide for pemphigoid-related airway obstruction, from which she died. No patients developed secondary malignancies.

Table 2.2 Adverse side effects

Drug	Status	n (%)	Adverse effect
Dapsone (n=90; 2852 patient months)	Discontinued	28 (31%)	Anaemia (8), rash (9), malaise (6), headache (2), diarrhoea (2), elevated LFTs (2), thrombocytopenia (1), dysplastic blood film (1), paraesthesia (2), back pain (1), stomach cramps (1), worsening of tinnitus (1)
	Continued	5 (5%)	Anaemia (5)
	Total	33 (37%)	
Sulfapyridine or sulfasalazine (n=48; 514 patient months)	Discontinued	12 (25%)	Rash (5), nausea & vomiting (3), malaise (1), depression (1), elevated LFTs & indigestion (1), lymphopenia (1), worsening of tinnitus (1), dizziness (1)
	Continued	3 (6%)	Nausea (2), anaemia (2)
	Total	15 (31%)	
Ciclosporin (n=7; 65 patient months)	Discontinued	2 (29%)	Elevated creatinine (2), hypertension (1)
	Continued	2 (29%)	Elevated creatinine (2), hypertension (1)
	Total	4 (57%)	
Methotrexate (n=4; 58 patient months)	Discontinued	1 (25%)	Elevated LFTs (1)
Prednisolone (n=55; 143 patient months short tapering course, 988 patient months <20mg/day low dose)	Discontinued	4 (7%)	Osteoporosis (2), hallucinations (1), malaise (1)
	Continued	1 (1%)	Peptic ulcer (1)
	Total	5 (9%)	
Azathioprine (n=60; 1230 patient months)	Discontinued	24 (40%)	Nausea & vomiting (8), headaches (4), elevated LFTs (3), malaise (3), dizziness (2), tinnitus (1), depression (1), diarrhoea (1), myelosuppression (1), itch (1), arthralgia (1), myalgia (1), chest & back pain (1), numbness (1), shivering spells (1)
	Continued	2 (3%)	Dizziness (1), abnormal blood results (1)
	Total	26 (43%)	
Mycophenolate (n=34; 506 patient months)	Discontinued	5 (15%)	Malaise (2), diarrhoea (1), rash (1), headache (1), shortness of breath & tremor (1)
	Continued	4 (12%)	Elevated LFTs (1), anaemia (1), myelosuppression (1), muscle cramps & insomnia (1)
	Total	9 (26%)	
Cyclophosphamide (n= 55; 512 patient months)	Discontinued	17 (31%)	Lymphopenia (5), lethargy & malaise (3), nausea & vomiting (2), diarrhoea (1), abdominal discomfort (2), anorexia & weight loss (2), severe pancytopenia (2), anaemia (1), elevated LFTs (1), rash (2), headache (1), insomnia (1), myalgia (1), unsteady on feet (1), shortness of breath (1), dysuria (1)
	Continued	10 (18%)	Lymphopenia (5)
	Total	27 (49%)	

LFTs = liver function tests. Anaemia, haemoglobin <13.3 g/dl (males) and <12.0 g/dl (females). Lymphopenia, $<0.5x10^9$/l.

2.4.7 Cicatrisation

Conjunctival cicatrisation at presentation was Mondino stage I in 13%, II in 43%, III in 28% and IV in 16% of eyes, and Foster stages I through IV in 2%, 15%, 81% and 2% of eyes, respectively. Progression of Mondino stage occurred in 53% of patients (61/115) and 41% of eyes (92/223), whilst Foster stage progressed in 8% of patients (9/115) and 4% of eyes (9/223). Using Mondino's staging system, progression occurred in 63% (19/30) stage I eyes, 48% (45/93) stage II eyes, 43% (25/58) stage III eyes and none of the stage IV eyes. Using Foster's staging system, progression occurred in 20% (1/5) stage I eyes, 18% (6/33) stage II eyes, 1% (2/180) stage III eyes and none of the stage IV eyes. There was evidence that progression of cicatrisation in the worst eye was associated with less severe inflammation (P = 0.0007) and longer duration of disease (P = 0.0048) (**Table 2.3**).

2.4.8 Visual acuity

The best corrected visual acuity at presentation was better than 6/18 in 67% of eyes, 6/18 to 6/60 in 16%, 3/60 to 6/60 in 5% and less than 3/60 in 11%. The final best corrected visual acuity was better than 6/18 in 48%, 6/18 to 6/60 in 19%, 3/60 to 6/60 in 7% and less than 3/60 in 26% of eyes. Ocular MMP was the cause of blindness (acuity less than 3/60) in 8% of eyes at presentation, and in 21% of eyes at the final visit. During the course of follow-up, the proportion of eyes with visual acuity 6/60 or less due to ocular MMP increased from 13% (29/223) to 26% (57/223); whilst the proportion of eyes with visual acuity 6/60 or less from other causes (retinal vascular event, glaucoma, macular degeneration) increased from 4% (8/223) at presentation to 7% (16/223) at the final visit. The best recorded acuity often required contact lens correction, but not all patients could tolerate contact lenses so a greater proportion of eyes had worse functional visual acuities.

Table 2.3 Progression of cicatrisation while receiving immunosuppressive therapy

	Total patients	No progression	Progression	P
No. of patients	115	54 (47%)	61 (53%)	
Duration of disease (mths)	51 (6-300)	43 (6-204)	68 (7-300)	0.0048*
Max inflammation grade 4	22	19 (86%)	3 (14%)	
Max inflammation grade 3	63	31 (49%)	32 (51%)	
Max inflammation grade 2	29	11 (38%)	18 (62%)	
Max inflammation grade 0 or 1	1	0	1 (100%)	0.0007*
Final acuity b	36	21 (58%)	15 (42%)	
Final acuity svi	8	4 (50%)	4 (50%)	
Final acuity vi	18	10 (56%)	8 (44%)	
Final acuity n	53	26 (49%)	27 (51%)	
Predominant outcome S	86	43 (50%)	43 (50%)	
Predominant outcome QS	23	15 (65%)	8 (35%)	
Predominant outcome F	6	3 (50%)	3 (50%)	
Final inflammation present	31	19 (61%)	12 (39%)	
Continuing therapy at last visit	65	33 (51%)	32 (49%)	
Biopsy positive	43†	27 (63%)	16 (37%)	
Extraocular disease present	58	35 (60%)	23 (40%)	

Progression defined by an increase in Mondino stage in the worst eye between initial and final visit. *Significant at P<0.01. n(%). Worst eye of each patient selected according to the following criteria: highest Mondino stage, worst inflammation, worst visual acuity, or OD eye if both eyes equal. Duration of disease, median (range). Max inflammation = maximum recorded level of inflammation. b = blind, svi = severe visual impairment, vi = visual impairment, n = normal. Predominant outcome = the outcome present for the greatest duration of the treatment course. S = success, QS = qualified success, F= failure. †Percentage of patients with biopsy results available.

2.5 Discussion

Although the final outcome was a quiet white eye in 78.5% (175/223) of eyes and in 70% (81/115) of patients, there was evidence of progressive cicatrisation in 41% (92/223) of eyes and 53% of patients (61/115). In half of this group of 92 eyes (n=46), progressive cicatrisation was associated with ongoing minimal to mild inflammation which immunosuppressive therapy was unable to completely eliminate, or when treatment was discontinued for various reasons, including toxicity. In contrast, the other 46 eyes with progression were reported as quiet and white. Interestingly, the analysis in **Table 2.3** also indicates that there seemed to be an association between less severe inflammation during the period of follow-up, and progression of cicatrisation.

However, the maximum recorded level of inflammation, upon which this analysis is based, was dependent first on the level of inflammation being accurately recorded in the case notes, which may or may not have been accurate on every occasion, and second on the maximum level of inflammation being present when the patient attended for their visit. Furthermore, detection of progression depended on observing an increase in Mondino stage, and this was not recordable for those who started at Mondino stage 4; in contrast, progression was more likely to be detected in those commencing at a lower Mondino stage, and 73% (22/30) of the patients with less severe inflammation (up to grade 2) commenced at Mondino stage 1 or 2. For these reasons, one should be cautious about making conclusions from this analysis. However, it could suggest that more aggressive treatment given to patients with severe inflammation also more effectively prevented progression of cicatrisation, unlike less aggressive, less toxic treatment given for less severe inflammation. Similar progression of cicatrisation despite reduction of inflammation has been reported previously (Elder *et al.*, 1996a; Letko *et al.*, 2004; Mondino & Brown, 1983).

Adverse side effects or the maximum safe duration of therapy to avoid adverse effects were the reason for changes in immunosuppressive therapy in 37% of treatment episodes (101/272) (**Table 2.1**). This indicates that the tolerability of immunosuppressive therapy in this elderly population of patients is limited, and the ability of physicians to use aggressive treatment doses and the most toxic agents to fully and completely suppress inflammation for prolonged durations is restricted.

Tauber et al (Tauber *et al.*, 1991) described results of a sequential regimen involving initial treatment of mild to moderate disease with dapsone, treatment of highly active disease with cyclophosphamide, and use of azathioprine for patients who did not respond to dapsone. Disease was brought under control in 92% of patients after additions or substitutions in regimens, and progression of scarring using the Foster staging system was detected in 6% of eyes and 10% of patients. We found that only 8% of patients in our study progressed according to the Foster staging system, whereas progression was evident in 53% of patients using Mondino staging. Another retrospective review similarly reported 10% progression using Foster staging in a cohort of patients with 90% control of inflammation (Miserocchi *et al.*, 2002); the results using the Mondino staging system reflect its increased sensitivity compared with the Foster system.

The proportion of eyes with visual acuity of 6/60 or less because of MMP doubled from 13% (29/223) to 26% (57/223) by the end of the study. This indicates that although control of inflammation may delay or prevent the onset of surface failure, it is not adequate to eliminate loss of vision due to irreversible corneal vascularisation and scarring as a consequence of recurrent epithelial defects and microbial keratitis.

2.6 Conclusion

Conventional immunosuppressive therapy using a stepladder regimen seems to be partially or completely successful in controlling inflammation to achieve a quiet white eye in three quarters of ocular MMP patients. However, there was evidence of progressive cicatrisation in half the patients. The extent to which maximum immunosuppressive therapy can be given is limited by its toxicity in this elderly patient group, and control of inflammation while limiting toxicity may not necessarily correlate with control of cicatrisation. More aggressive immunosuppressive treatment could more effectively prevent progression of cicatrisation, and more rapid control of inflammation could potentially be more effective at arresting cicatrisation.

Chapter 3

A pilot randomised controlled trial of pulse intravenous methylprednisolone in severe ocular mucous membrane pemphigoid

3.1 Introduction

The findings in chapter 2 indicate that our current conventional immunosuppressive therapy regimen does not arrest cicatrisation in just over half the patients. We hypothesise that more aggressive treatment and more rapid control of inflammation could more effectively prevent progression.

It has been observed that systemic corticosteroids prevent conjunctival shrinkage and fibrosis in ocular MMP, when used in the setting of acute severe inflammation (Mondino & Brown, 1981). However, it is well recognised that systemic corticosteroid treatment alone is not appropriate treatment for ocular MMP, given that the high steroid doses required to sustain disease control are associated with significant morbidity and mortality in this elderly patient group, and because the inflammation recurs when the systemic corticosteroids are tapered (Hardy *et al.*, 1971).

High dose intravenous methylprednisolone (IVMP) pulse therapy may be an effective additional measure to reduce the time to control of inflammation, in combination with cyclophosphamide and oral corticosteroids in ocular MMP. More rapid control of inflammation could more effectively suppress aggressive inflammation, and perhaps also delay cicatrisation. Pulse IVMP is used anecdotally in clinical practice by physicians managing ocular MMP. It has been reported to be beneficial in an uncontrolled case series of severe inflammatory eye disease (Wakefield *et al.*, 1986), and its use in ocular MMP has been mentioned (McCluskey *et al.*, 2004; Sacher & Hunzelmann, 2005), but not formally investigated. There is good evidence of the efficacy of pulse IVMP treatment in other inflammatory conditions such as optic neuritis, where pulse intravenous methylprednisolone (IVMP) followed by oral prednisone speeds the recovery of visual loss compared with oral prednisone alone (Beck *et al.*, 1992). Pulse IVMP has also been shown in randomised controlled trials to give rapid temporary relief

of flares in rheumatoid arthritis, with clinical improvement lasting 6 weeks or longer (Liebling *et al.*, 1981; Williams *et al.*, 1982).

In a study of severely inflamed ocular MMP patients by Elder et al (Elder *et al.*, 1995), using conventional treatment of oral cyclophosphamide and oral corticosteroids, ocular inflammation resolved in 26% (5/19 eyes) and 30% (3/10) patients by 6 weeks. In a study where the pre-treatment conjunctival inflammation may not have been as severe, Foster reported that inflammation had resolved and the eyes were white and quiet in 50% (6/12) of patients by 6 weeks (Foster, 1986). We therefore hypothesised that the proportion of patients with control of inflammation at 6 weeks could be significantly increased by pulse IVMP.

3.2 Aim

To date there have been only 2 prospective randomised trials evaluating treatment for mucous membrane pemphigoid (Foster, 1986); all other reports are interventional case series or cohort studies (Saw *et al.*, 2008). The purpose of the study in this chapter was to establish the feasibility of conducting a randomised controlled clinical trial to evaluate whether there is any additive effect of pulse intravenous methylprednisolone, in reducing the time to control of inflammation in patients with severely active ocular MMP who are commencing oral corticosteroid and cyclophosphamide treatment, and to evaluate whether more rapid control of inflammation also more effectively arrests cicatrisation.

3.3 Research design and specific methods

I designed and coordinated a multi-centre pilot randomized controlled clinical trial which was conducted according to the guidelines of the Declaration of Helsinki. Patients were recruited from September 2005 through April 2008. The Moorfields Eye Hospital Research Governance Committee and the Oxfordshire Research Ethics Committee approved the study protocol, and informed consent was obtained

from all participants (see **Appendix 3**). The trial is registered on the International Standard Randomised Controlled Trial Number Register (ISRCTN51714283).

3.3.1 Participants

Inclusion criteria were as follows: patients with clinical features consistent with ocular MMP i.e. progressive conjunctival cicatrisation and inflammation where other causes of conjunctival scarring have been excluded, with or without a positive result on direct immunofluorescence (DIF) microscopy from any mucosa or skin biopsy showing linear basement membrane deposition of IgG, IgA, and/or C3, and/or a positive result on indirect immunofluorescence (IIF) testing for anti-basement membrane zone antibodies. Presence of a clinical indication to commence cyclophosphamide therapy because of bilateral or unilateral 'moderate' (grade 3) or 'severe' (grade 4) ocular inflammation and/or active conjunctival inflammation uncontrolled by previous non-cyclophosphamide immunosuppression was a criterion for inclusion.

Exclusion criteria included patients already receiving cyclophosphamide, patients with other causes of progressive conjunctival scarring (drug-induced pemphigoid with negative direct immunofluorescence biopsy, atopic keratoconjunctivitis, Sjogren's syndrome, Stevens-Johnson syndrome, chemical injury), active secondary malignancy, HIV infection, pregnancy or breastfeeding.

The study eye was chosen in each patient based on the eye with the greatest conjunctival inflammation at enrolment; where both eyes had similar inflammation, the right eye was chosen.

3.3.2 Recruitment

Patients were recruited as consecutive cases from the clinic population in 3 centres: Moorfields Eye Hospital (n=16), Birmingham and Midlands Eye Centre

(n=3) and Bristol Eye Hospital (n=1). Study investigators and masked observers were chosen at each site.

3.3.3 Intervention

The **intervention** given to the treatment group was adjunctive intravenous methylprednisolone (IVMP) 1 gram daily for 3 consecutive days. Oral cyclophosphamide 1.5 mg/kg/day was commenced simultaneously, and a 12 week tapering course of oral prednisolone was commenced after the 3rd day of IVMP. The oral prednisolone dosage was 1mg/kg/day for 1 week, reducing by 10mg/ week until the dose was 60mg/day. Following this, the dose was reduced by 5mg each week and the treatment reduction was planned to result in a dose of 5 to 7.5mg /day at week 12, then continued at 5 to 7.5mg/d for a further 3 months then ceased. At the week 4 visit, if the conjunctival inflammation was still grade 2 or above in each of the 4 quadrants (see Outcome Measures below), a second pulse of 3 grams IVMP was given.

The control treatment was conventional therapy with oral cyclophosphamide 1.5 mg/kg/day and a 12 week tapering course of oral prednisolone 1mg/kg/day. In all patients, cyclophosphamide was continued for up to 1 year providing no adverse events necessitated its discontinuation. At the end of 12 months cyclophosphamide was replaced with an alternative, less toxic immunosuppressant i.e. stepping down on the step-ladder algorithm of immunosuppressive therapy (see Chapter 1, **Figure 1.1**). All patients were commenced on alendronic acid 70mg weekly with oral calcium and vitamin D3, and omeprazole or a similar anti-dyspepsia agent until the oral corticosteroid doses had reduced to 7.5mg or less per day.

3.3.4 Randomization and Masking

A randomisation list was generated by the statisticians at the Clinical Trials Unit (CTU) at Moorfields, using random permuted blocks of varying sizes. This list was kept at the CTU and when a patient required allocation to treatment, the CTU were

contacted, the patient was registered with the CTU, and then assigned their treatment. Masked observers at each study site graded conjunctival inflammation at each visit and the cicatrising conjunctivitis severity score at enrolment and the final visit. Masked photographic grading of conjunctival inflammation at each visit was carried out in parallel at the Moorfields Eye Hospital Reading Centre. Study investigators and patients were not masked, and study investigators were informed of the treatment allocation by the biostatistician via a central telephone number at the time of randomization. The success of masking was maintained by ensuring that the masked observers were excluded at all times from access to the study folder which contained the treatment details, and they did not discuss treatment with the patient. The masked photographic grader did not have any access to information about the treatment allocation.

3.3.5 Outcome Measures

Assessment tools for grading the outcome measures of conjunctival inflammation, fornix depth and severity of cicatrising conjunctivitis were created, based on modifications of previously described tools.

The **primary outcome measure** was the proportion of patients achieving control of conjunctival inflammation in the most inflamed eye (the study eye) at 6 weeks. Control of conjunctival inflammation was defined as bulbar conjunctival inflammation grade 1.5 or less in all 4 quadrants (upper nasal, lower nasal, upper temporal, lower temporal), giving a total bulbar inflammation score of 6 or less, out of a potential total score of 16. Grading of conjunctival inflammation in 4 quadrants was carried out because sectoral variation in conjunctival inflammation is often observed in ocular MMP, making it difficult to give an “overall” grade when the inflammation could be very severe in one quadrant, but minimal in another. Conjunctival inflammation was graded from grade 0 (nil), grade 1 (minimal), grade 2 (moderate), grade 3 (marked), and grade 4 (severe), as described previously (Elder *et al.*, 1995; Foster, 1986). For grading conjunctival inflammation, a grading

sheet showing representative photographs of each grade from 0 to 4 was created (see **Figure 3.1**).

This grading sheet was used by the masked observers and the photographic grader. Validation of this grading sheet for assessing conjunctival inflammation was carried out prior to commencing the study, using 3 observers in 10 sample patients with different grades of conjunctival inflammation, assessing inter-observer and test-retest reliability. Although other grading scales for conjunctival inflammation related to contact lens wear are in use e.g. the CCLRU grading scale (Efron *et al.*, 2001), the severity of grade 4 conjunctival inflammation in ocular MMP is much more severe than in any of these grading scales, so an alternative disease-appropriate grading scale was devised by selecting suitable photographs of ocular MMP. Conjunctival inflammation was graded at 2 weekly intervals for 3 months, 4 weekly for 3 months, then 8 weekly for 6 months i.e. a total of 13 study visits over 12 months.

Secondary outcome measures included time to control of inflammation in weeks, additional therapy required after week 12 for inflammatory control, progression of cicatrisation at 12 months or their final visit when this was earlier, change in cicatrising conjunctivitis severity score, visual acuity, and adverse side effects including change in bone densitometry.

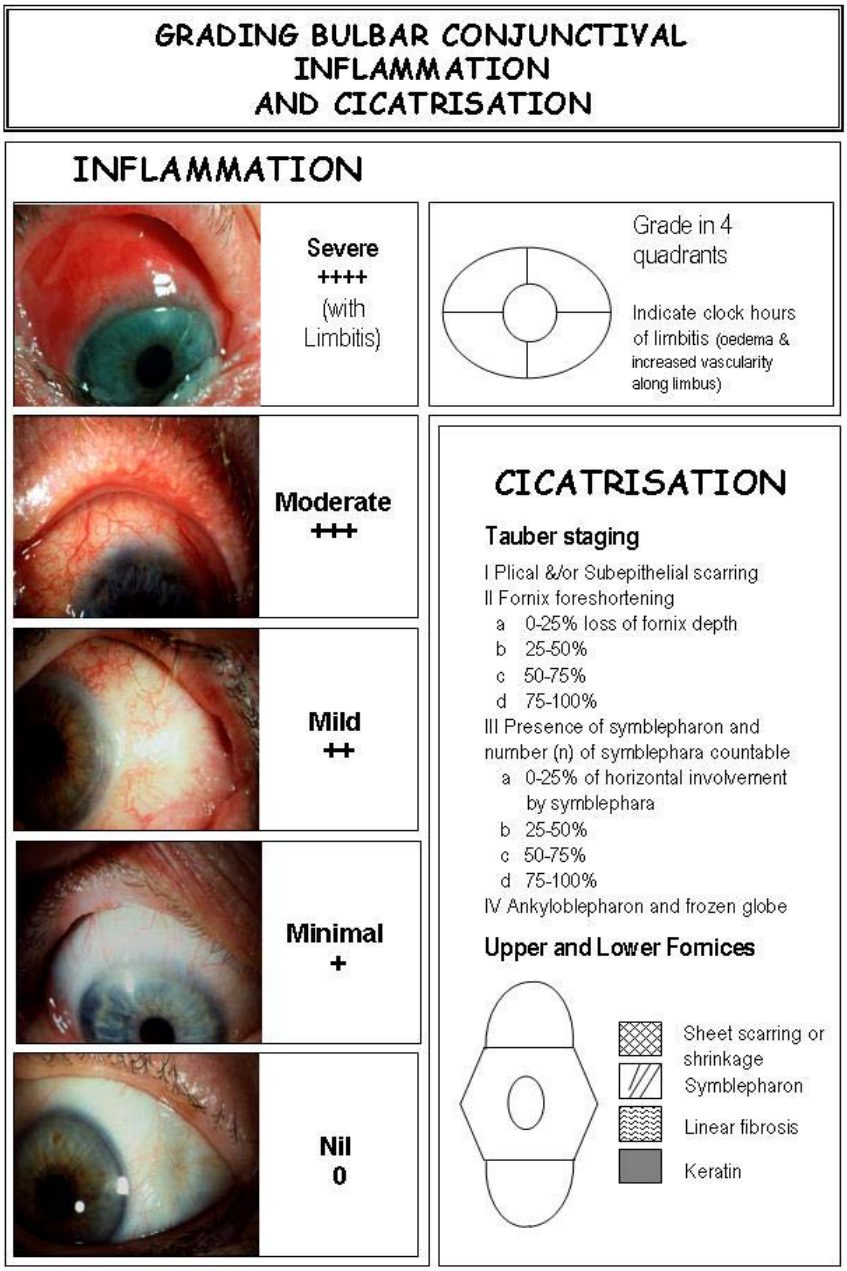


Figure 3.1 Grading sheet for bulbar conjunctival inflammation and cicatrisation.

Evaluating progression of cicatrisation: developing a conjunctival fornix measuring instrument

Progression of cicatrisation between the enrolment and 12 month visit was evaluated by Tauber staging of ocular cicatrisation (Tauber *et al.*, 1992) by slit lamp exam and using a custom-made fornix measuring instrument (**Figure 3.2**). The design of the fornix measuring instrument was based on that of a previously published device (Schwab *et al.*, 1992), and adapted by us (myself, Scott Hau: senior optometrist at Moorfields Eye Hospital and David Carpenter: ocular prosthetist at Moorfields Eye Hospital) to enable measurement of the upper conjunctival fornix. Schwab et al have demonstrated that this type of fornix measuring instrument has good inter-rater as well as test-retest reliability. The device, used with topical anaesthesia and disinfected with hypochlorite between patients, permits rapid measurement and does not cause any discomfort or pain.

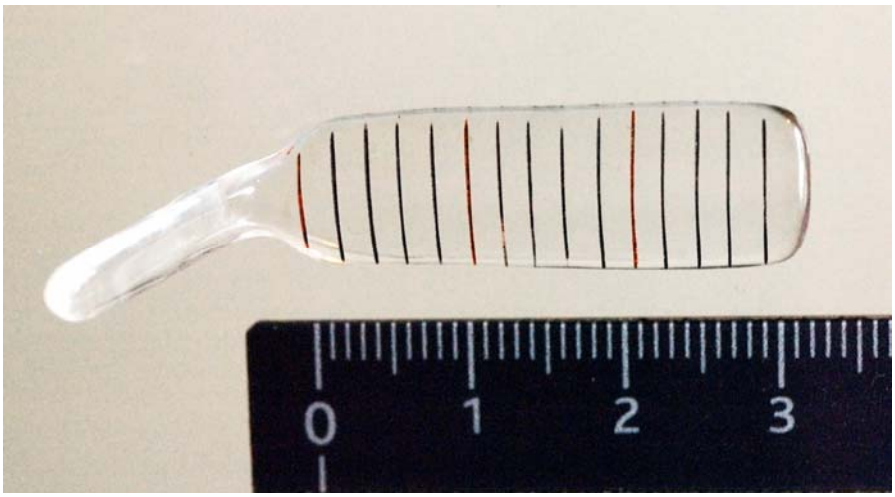


Fig 3.2 Fornix measuring instrument custom-designed to enable measurement of the upper and lower conjunctival fornices. The design of this device was an adaptation of a similar instrument described by Schwab et al (Schwab *et al.*, 1992).

Evaluating progression of cicatrisation: investigating what is normal upper conjunctival fornix depth

Tauber staging of fornix shortening is dependent on knowledge of the normal fornix depth (see **Figure 3.1**): Stage IIa is 0-25% loss of fornix depth, IIb is 25-50%, IIc is 50-75%, IId is 75-100%. Whilst the normal lower conjunctival fornix depth has been evaluated and found to be 10-11mm for normal subjects aged 50 years or older (Schwab *et al.*, 1992), with 10mm being the average fornix depth in subjects aged 80 years or older, the normal upper conjunctival fornix depth is unknown. At the time of commencement of the trial in 2005, information about normal upper conjunctival fornix depth could not be found in any major ophthalmology reference books or by a literature search on Pubmed or google using the term “conjunctival fornix depth” or “conjunctival fornix measurement”.

Almost all published studies in ocular MMP report only lower conjunctival fornix staging; the upper conjunctival fornix is often overlooked. McCluskey et al reported the upper conjunctival fornix cicatrisation staging in their patients, but it is unclear how they established, for example, that a patient had an upper fornix stage IIc (McCluskey *et al.*, 2004). Asymmetrical severity of involvement between right and left eyes is a recognised feature of ocular MMP (Mondino & Brown, 1983), and it could be expected that similar asymmetry might occur between the upper and lower conjunctival fornices of each eye. For this reason, it was felt that evaluation of both lower and upper conjunctival fornices was the most accurate way to stage conjunctival cicatrisation, and that this was imperative in any prospective studies evaluating progression of conjunctival cicatrisation.

Having designed an instrument that enabled us to measure upper conjunctival fornix depth, it was necessary to investigate and try to establish what range of measurements would be considered normal upper conjunctival fornix depth, in eyes with healthy conjunctiva. Approval from the Moorfields and Whittington Ethics committee was sought to commence a study where the aim was to establish what

range of measurements constitute normal upper conjunctival fornix depth (see **Appendix 4**). Based on preliminary results from 127 patients aged 50 years and older, the average upper conjunctival fornix depth was 15.2 ± 1.6mm, range 12 – 20mm (see **Appendix 4**). These results were used to determine upper conjunctival Tauber staging (Tauber IIa 12-20mm, IIb 8- 11mm, IIc 4- 7mm, IId <4mm).

A **cicatrising conjunctivitis severity score** was devised, based on previous grading schemes (Elder & Bernauer, 1997; Francis *et al.*, 1990). This evaluated visual acuity, conjunctival inflammation, lid pathology and cicatrisation as well as keratopathy, to give an indication of the severity of disease affecting all aspects of the ocular surface (see **Figure 3.3**). The cicatrising conjunctivitis severity score was assessed by the masked observers at enrolment and at the final visit, for each patient.

Adverse side effects were recorded at each visit. To detect toxicity due to immunosuppressive therapy, the following parameters were monitored in all patients at commencement of therapy and at each study visit: blood pressure, pinprick blood sugar, dipstick urinalysis, full blood count, renal function and electrolytes, liver function tests. The cyclophosphamide dose was titrated to achieve a target lymphocyte count level is 0.5-1.0 x 10^9/L, aiming for lymphopenia without leukopenia (white cell count >3.0 x 10^9/L, neutrophils >2.0 x 10^9/L). Patients were admitted to hospital for the IVMP infusions, and their electrocardiograph, vital observations, blood sugar levels, full blood count, renal function and electrolytes, liver function tests were monitored. Bone densitometry was performed at enrolment and at the final visit. Conjunctival biopsies and blood samples were also taken for concurrent laboratory studies.

CICATRISING CONJUNCTIVITIS SEVERITY SCORE

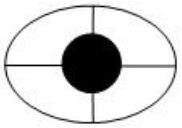
Best corrected visual acuity

1= 6/6	6= 6/36	11= PL	
2= 6/9	7= 6/60	12= NPL	
3= 6/12	8= 3/60	98= not applicable	correction method…….
4= 6/18	9= 1/60	99= unknown	
5= 6/24	10= HM, CF		

Score /3

BULBAR INFLAMMATION IN EACH QUADRANT

0 = nil
0.5 to 1.0 = minimal
1.5 to 2.5= mild
3.0 to 3.5= moderate
4= severe



Score /16

Limbitis
0= absent 1= present (specify clock hours)……

Score /1

Mucus
0=nil 1=mild 2= severe ……

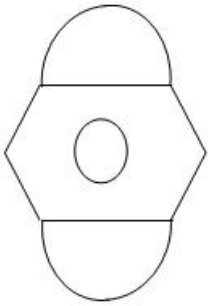
Score /2

Schirmer's (without anaesthetic) at 5 min (mm)

Score /3

CONJUNCTIVAL CICATRISATION

Sheet scarring or shrinkage
Symblepharon
Linear fibrosis
Keratin



Keratinisation 0= nil 1= mild 2= prominent

Score /2

Trichiasis & Metaplastic lashes 0= nil 1= 1-3 lashes 2= 4-6 lashes 3= >7 lashes

/3

Entropion 0= absent 1= One lid 2= Both lids

/2

Lagophthalmos due to OCP 0= absent 1= present

/1

Reduced Ocular rotations
0= normal 1= 1 direction 2= 2 directions 3= >2 directions 4= no movement

/4

Upper fornix measure (mm)
Lower fornix measure (mm)

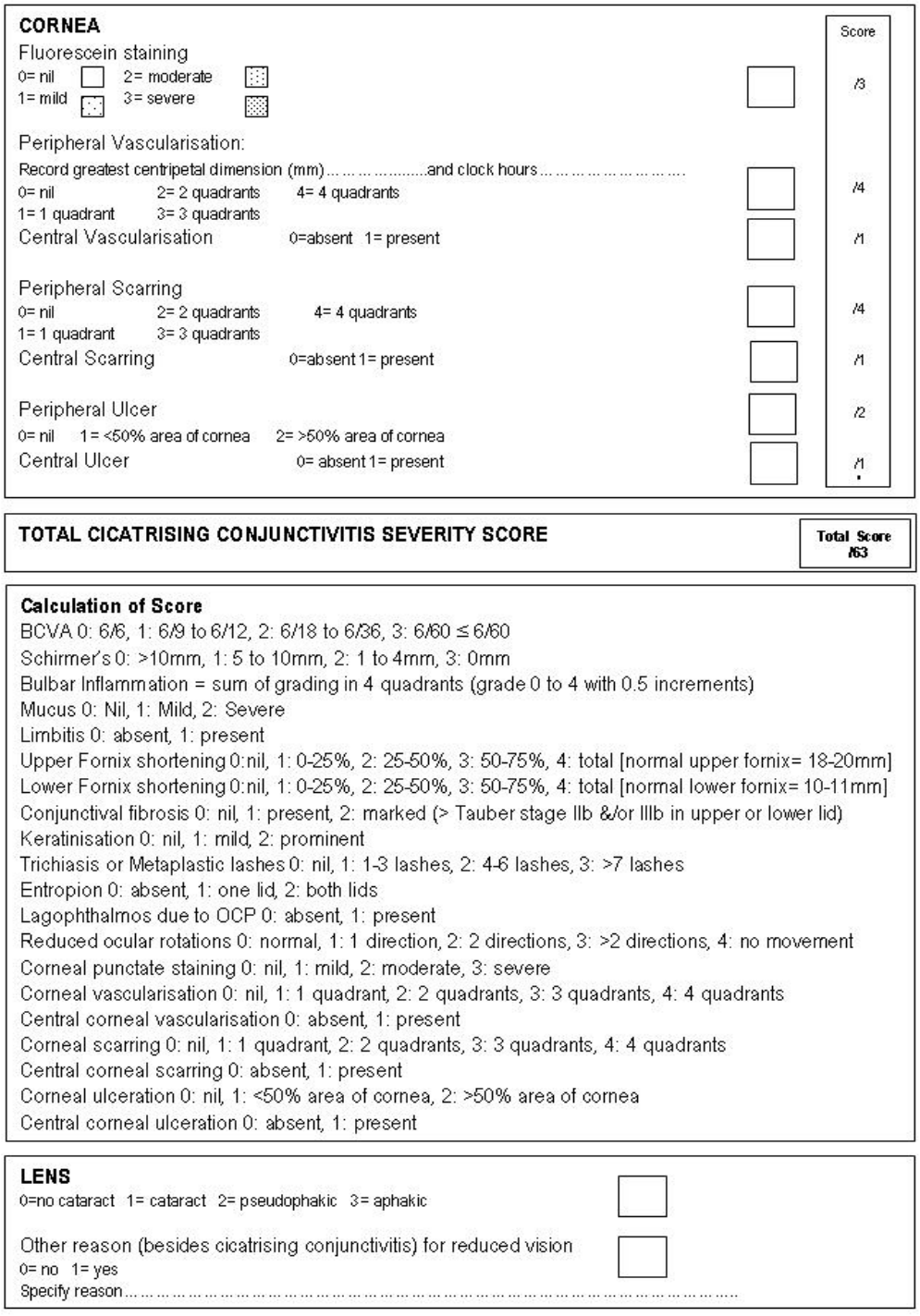
Score /4 /4

Upper Tauber Stage ……

Lower Tauber Stage ……

Conjunctival fibrosis 0=nil 1= present 2= marked (>Tauber stage IIb &/or IIIb in either lid)

Score /2



CORNEA

Score

Fluorescein staining
0= nil 2= moderate
1= mild 3= severe
/3

Peripheral Vascularisation:
Record greatest centripetal dimension (mm)……..and clock hours……
0= nil 2= 2 quadrants 4= 4 quadrants
1= 1 quadrant 3= 3 quadrants
/4

Central Vascularisation 0=absent 1= present
/1

Peripheral Scarring
0= nil 2= 2 quadrants 4= 4 quadrants
1= 1 quadrant 3= 3 quadrants
/4

Central Scarring 0=absent 1= present
/1

Peripheral Ulcer
0= nil 1= <50% area of cornea 2= >50% area of cornea
/2

Central Ulcer 0= absent 1= present
/1

TOTAL CICATRISING CONJUNCTIVITIS SEVERITY SCORE

Total Score /63

Calculation of Score

BCVA 0: 6/6, 1: 6/9 to 6/12, 2: 6/18 to 6/36, 3: 6/60 ≤ 6/60
Schirmer's 0: >10mm, 1: 5 to 10mm, 2: 1 to 4mm, 3: 0mm
Bulbar Inflammation = sum of grading in 4 quadrants (grade 0 to 4 with 0.5 increments)
Mucus 0: Nil, 1: Mild, 2: Severe
Limbitis 0: absent, 1: present
Upper Fornix shortening 0:nil, 1: 0-25%, 2: 25-50%, 3: 50-75%, 4: total [normal upper fornix= 18-20mm]
Lower Fornix shortening 0:nil, 1: 0-25%, 2: 25-50%, 3: 50-75%, 4: total [normal lower fornix= 10-11mm]
Conjunctival fibrosis 0: nil, 1: present, 2: marked (> Tauber stage IIb &/or IIIb in upper or lower lid)
Keratinisation 0: nil, 1: mild, 2: prominent
Trichiasis or Metaplastic lashes 0: nil, 1: 1-3 lashes, 2: 4-6 lashes, 3: >7 lashes
Entropion 0: absent, 1: one lid, 2: both lids
Lagophthalmos due to OCP 0: absent, 1: present
Reduced ocular rotations 0: normal, 1: 1 direction, 2: 2 directions, 3: >2 directions, 4: no movement
Corneal punctate staining 0: nil, 1: mild, 2: moderate, 3: severe
Corneal vascularisation 0: nil, 1: 1 quadrant, 2: 2 quadrants, 3: 3 quadrants, 4: 4 quadrants
Central corneal vascularisation 0: absent, 1: present
Corneal scarring 0: nil, 1: 1 quadrant, 2: 2 quadrants, 3: 3 quadrants, 4: 4 quadrants
Central corneal scarring 0: absent, 1: present
Corneal ulceration 0: nil, 1: <50% area of cornea, 2: >50% area of cornea
Central corneal ulceration 0: absent, 1: present

LENS

0=no cataract 1= cataract 2= pseudophakic 3= aphakic

Other reason (besides cicatrising conjunctivitis) for reduced vision
0= no 1= yes
Specify reason……

Figure 3.3 Cicatrising Conjunctivitis Severity Score

3.3.6 Sample Size

With conventional treatment of oral cyclophosphamide and oral corticosteroids there is a 26% (5/19 eyes) success rate at 6 weeks (Elder *et al.*, 1995). On this basis, 9 eyes per treatment group would give 80% power to detect a treatment difference of 70%, at the 5% level (Fleiss, 1981). This does not include any adjustment for loss to follow−up because the study duration is short and the condition is severe.

3.3.7 Statistical Analysis

Baseline characteristics of the treatment groups were compared to assess the adequacy of randomization. Descriptive statistics were used because of the small patient numbers. The number of patients required for a definitive study was computed. Agreement between photographic and observer grading of bulbar inflammation was assessed using Bland Altman methods. Analysis was by intention to treat.

3.4 Results

3.4.1 Participants

Of the 40 patients assessed for eligibility in the study, 18 declined to participate, 1 did not meet the inclusion criteria as she had already been commenced on cyclophosphamide, and 1 was excluded because he had severe disease that the study investigator at that site wished to treat with IVMP and subsequently rituximab. The main reasons given by patients for declining to participate included the inconvenience, distance to travel, having domestic commitments to care for a disabled relative, and not wishing to be randomised to IVMP because of concomitant illnesses like diabetes. The remaining 20 patients were randomised to either IVMP or the control group. **Figure 3.4** shows a flowchart of the progress of subjects through the phases of the trial.

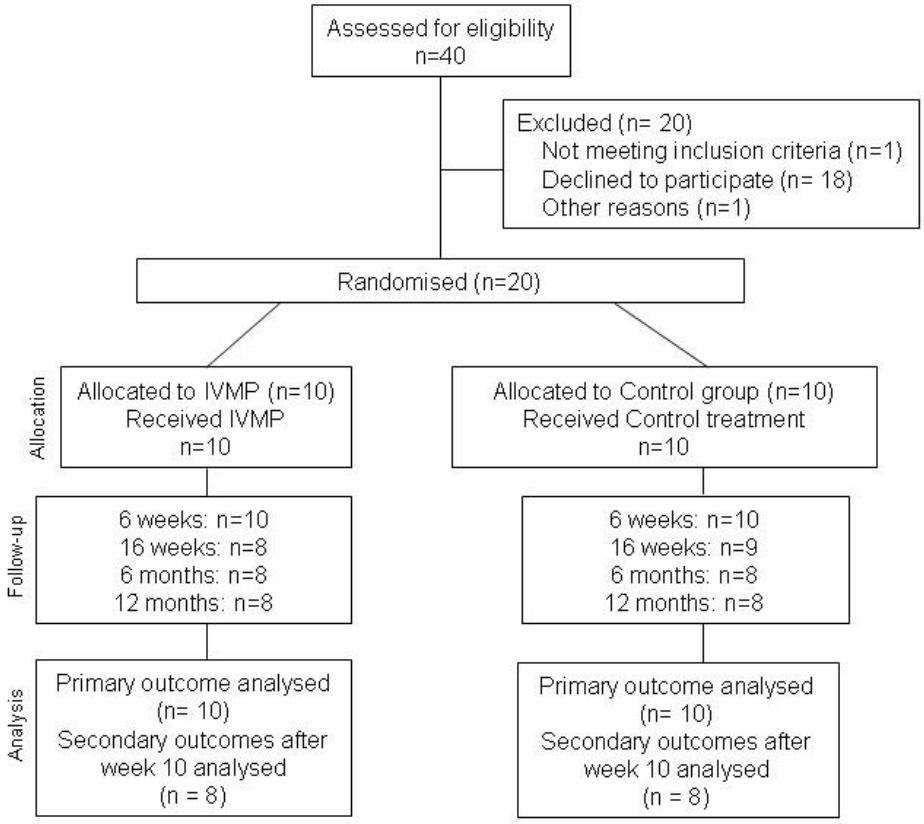


Figure 3.4 Flowchart of the progress of subjects through the phases of the trial

The primary outcome measure was analysed at 6 weeks for all 20 patients. Two patients in the IVMP group died: an 80 year old male who had received 3 grams of IVMP at enrolment died at week 16 of a pulmonary embolism which was thought to be unrelated to the study treatment; a 73 year old male died at week 10 of pneumonia and heart failure. The opinion of the physician managing the latter patient was that the IVMP was unlikely to have contributed to the development of heart failure. Two patients in the control group died: an 83 year old male died at week 12 of community-acquired pneumonia and a 73 year old male died at week 40 of metastatic lung carcinoma. The latter patient had previously been a heavy smoker and the carcinoma was diagnosed 5 months following enrolment in the study. It is likely that the systemic immunosuppression with both

cyclophosphamide and high dose corticosteroids was a predisposing factor for pneumonia in the 2 patients who developed this complication.

3.4.2 Comparability of IVMP group and control group

The 2 study groups were comparable in terms of disease duration, prior non-cyclophosphamide immunosuppressive therapy, any lid surgery prior to enrolment or during the study follow-up period, and use of topical corticosteroids (**Table 3.1**). The median age in the control group was younger (58 years) than that in the IVMP group (76 years) but this difference was not significant with these numbers. Mucous membrane graft fornix reconstruction was carried out on the study eye in 2 patients in the IVMP group (at weeks 24 and 39), and 1 patient in the control group (at week 26). Calculations of the bulbar inflammation scores following surgery in these patients excluded the quadrants involving the mucosal graft.

Prognostic variables in the IVMP group appeared to be slightly worse than those in the control group, suggesting that any definitive trial would need to stratify for such factors in the randomisation. The study eye in the IVMP group had a higher initial bulbar inflammation score (median 12.5) compared with the control group (median 11), more advanced cicatrisation (10/10 Tauber stage IIb or above and 10/10 Tauber stage IIIa or above in the IVMP group versus 7/10 and 8/10 respectively in the control group) as well as shorter upper and lower fornix depths and a higher initial cicatrising conjunctivitis severity score (**Table 3.2**).

Table 3.1 Patient characteristics

PATIENT CHARACTERISTICS	All (n=20)	IVMP (n=10)	Control (n=10)
Age (median, range)	61 (51 - 82)	76 (53 - 82)	58 (51 - 82)
Gender			
Male	14	8	6
Female	6	2	4
Eye			
Right	14	6	8
Left	6	4	2
IF positive	17	8	9
Extraocular Disease	10	4	6
Autoimmune Disease	2	2	0
Disease Duration in Years (median, range)	2 (0 - 18)	2 (0 - 10)	2 (3 - 18)
Any Lid surgery	6	3	3
Topical Steroids used during Trial	15	6	9
Prior non-cyclophosphamide immunosuppressive therapy	9	4	5

IVMP = intravenous methylprednisolone, IF = immunofluorescence

Table 3.2 Clinical features of the study eye

	All (n=20)	IVMP (n=10)	Control (n=10)
Initial Bulbar Inflammation Score (/ 16) (median, range)	11 (8.5 - 16)	12.5 (10 - 16)	11 (8.5 - 12.5)
Initial Tauber Stage (lower lid)			
II a	3	0	3
II b	4	4	0
II c	7	2	5
II d	6	4	2
Tauber stage IIb or greater		100% (10/10)	70% (7/10)
III a	5	1	2
III b	5	4	1
III c	3	0	3
III d	7	5	2
Tauber stage IIIa or greater		100% (10/10)	80% (8/10)
Fornix Depth (mm)			
Upper (median, range)	11 (4 - 21)	10 (4 - 20)	12 (4 - 21)
Lower (median, range)	4 (0 - 10)	3.5 (0 - 6)	4 (0 - 10)
Visual Acuity (LogMAR) (median, range)	0.18 (0.00 - 1.00)	0.18 (0.00 - 1.00)	0.30 (0.00 - 1.00)
Initial MMP Severity Score (/ 63) (median, range)	29 (15 - 43)	32.5 (19 - 43)	27 (15 - 39)

IVMP = intravenous methylprednisolone, MMP = mucous membrane pemphigoid

3.4.3 Primary outcome

The primary outcome measure, the proportion of patients with control of inflammation at 6 weeks, was 20% (2/10) in the IVMP group and 20% (2/10) in the control group (**Table 3.3**). A rapid response to IVMP was observed at 2 weeks, but the effect was short-lived and not maintained at 4 weeks. Additional therapy was required after week 12 due to poor inflammatory control in 33.3% (3/9 patients: rituximab in 2 patients, dapsone in 2 patients) and 30% (3/10: dapsone in all 3 patients) of the patients in the IVMP and control groups respectively. A rebound of inflammation following initial control (which was persistent despite addressing potential ocular surface causes of a flare of inflammation including blepharitis, dessication, exposure, trichiasis, and was therefore likely to be due to the underlying immune disorder) was observed in 75% (6/8) and 40% (4/10) of the IVMP and control groups respectively. The median time to control was 8 weeks in the IVMP group and 14 weeks in the control group. Representative plots of the response of bulbar inflammation to treatment over the 12 month follow-up period are shown in **Figure 3.5**.

The masked photographic grading scores of bulbar conjunctival inflammation tended to be higher than the masked observer scores, with larger differences observed where there was higher inflammation. In no case was disagreement greater than 5 points. This is shown in **Figure 3.6**.

An exploratory analysis suggested that factors associated with absence of control of inflammation at 6 months included a longer disease duration, a positive immunopathology result, any lid surgery, a higher initial cicatrising conjunctivitis severity score, and a longer duration to control of inflammation (**Table 3.4**).

Table 3.3 Control of inflammation at different time points

	All (n=20)	IVMP (n=10)	Control (n=10)
% controlled at 6 weeks	20% (4/20)	20% (2/10)	20% (2/10)
% controlled at 16 weeks	40% (8/20)	37.5% (3/8)	55.6% (5/9)
% of these controlled at 6 mths	71% (5/7)	100% (3/3)	75% (3/4)
% of these controlled at 12 mths	71% (5/7)	66.7% (2/3)	75% (3/4)
% controlled at 6 months	43.8% (7/16)	50% (4/8)	37.5% (3/8)
% controlled at 12 months	66.7% (10/15)	62.5% (5/8)	71.4% (5/7)
Median time to control (weeks) (range)	20 (2 - 52)	8 (6 - 52)	14 (2 - 48)
Additional therapy required after week 12 due to poor inflammatory control	31.5 (6/19)	33% (3/9)	30% (3/10)
Inflammation rebound after initial control	55.6% (10/18)	75% (6/8)	40% (4/10)

Table 3.4 Factors associated with control of inflammation at 6 months

	Controlled at 6 months (n=7)	Not controlled at 6 months (n=9)
Received IVMP	43% (3/7)	44% (4/9)
Age (median, range)	62 (51 - 82)	59 (53 - 76)
Immunopathology positive	71% (5/7)	89% (8/9)
Extraocular Disease	43% (3/7)	56% (5/9)
Autoimmune Disease	14% (1/7)	11% (1/9)
Disease Duration (years) (median, range)	0.25 (0.1 - 7)	2 (0 - 18)
Any Lid surgery	43% (3/7)	78% (7/9)
Topical Steroids used during Trial	71% (5/7)	89% (8/9)
Initial Bulbar Inflammation (median, range)	11 (8.5 - 14)	11 (8.5 - 16)
Initial Cic Conj Severity Score (median, range)	23.5 (15 - 27)	30.75 (17.5 - 43)
Fornix Depth- upper (mm) (median, range)	12 (10 - 16)	8.5 (3.5 - 21)
Fornix Depth- lower (mm) (median, range)	3 (1 - 10)	4 (0 - 10)
Median time to control (weeks) (range)	16 (6 - 24)	32 (2 - 52)

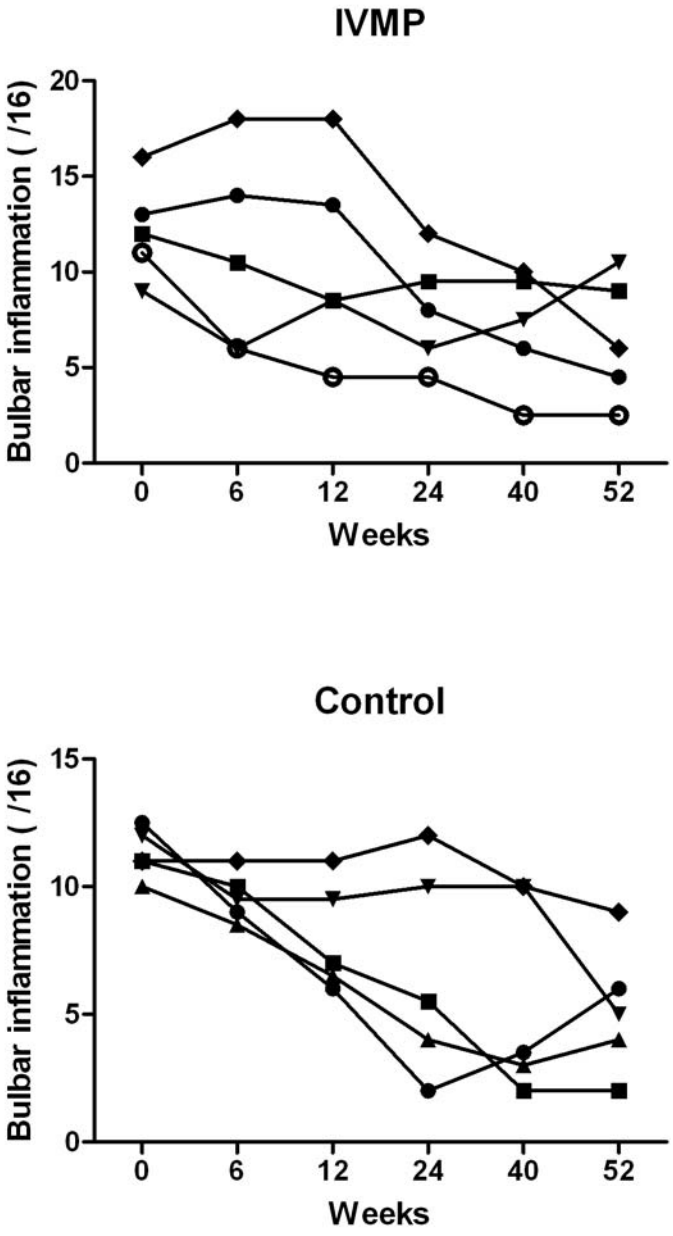


Figure 3.5 Change in bulbar inflammation over 12 months

Representative plots of the response of bulbar inflammation to treatment over the 12 month follow-up period, for 5 patients in the IVMP intravenous methylprednisolone group, and 5 patients in the control group.

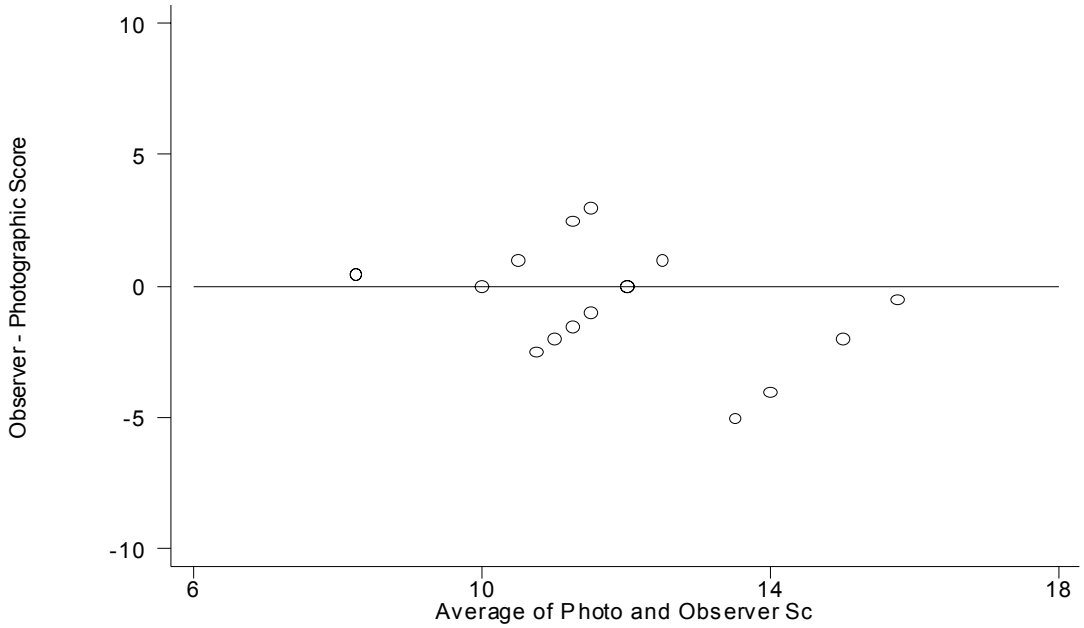


Figure 3.6 Bland Altman plot comparing the masked photographic score and masked observer score of bulbar inflammation. The Y axis shows the observer score minus the photographic score, the X axis shows the average of the photographic and observer scores. There are more points below the line than above, indicating a tendency for the photographic scores to be higher than the observer scores, with larger differences observed when there is greater inflammation.

3.4.4 Progression of cicatrisation

Of the 16 patients with 12 month follow-up data, there was a decrease in fornix depth of at least 2mm in 25% (2/8) of both the IVMP and control groups. An increase in the centripetal or circumferential extent of corneal pannus was detected in 75% (6/8) of the IVMP patients and 50% (4/8) of the control group patients (**Table 3.5**). Although the small patient numbers limit the ability to make conclusions from the data, there did not appear to be any correlation between time to control of inflammation and increase in fibrosis, but the median time to control of inflammation in patients without an increase in corneal pannus was shorter (week 10) than in patients who did have an increase in corneal pannus (week 26), suggesting that more rapid control of inflammation is associated with a reduced

likelihood of progression in corneal pannus. The cicatrising conjunctivitis severity score at 12 months improved (a reduction in the score) in all 16 patients apart from one patient in the IVMP group where it worsened. Best corrected visual acuity worsened in 3 patients and improved in 1 patient in the IVMP group, and improved in 2 patients in the control group; there was no change in the remaining patients. Characteristics of the patients in whom progression of fibrosis at 12 months was detected are described in **Table 3.6**.

Table 3.5 Progression of cicatrisation

	All (n=16)	IVMP (n=8)	Control (n=8)
Decrease in fornix depth	25% (4/16)	25% (2/8)	25% (2/8)
Time to control in patients with decrease in fornix depth	Week 16 (week 8 to 52)	Week 20, week 52	Week 12, week 8
Time to control in patients with no change in fornix depth (median, range)	Week 20 (week 2 to week 48)	Week 24 (week 6 to week 32)	Week 18 (week 2 to week 48)
Increase in corneal pannus	62.5% (10/16)	75% (6/8)	50% (4/8)
Time to control in patients with increase in pannus (median, range)	Week 26 (week 6 to week 52)	Week 32 (week 6 to week 52)	Week 20 (week 8 to week 48)
Time to control in patients with no change in pannus (median, range)	Week 10 (week 2 to week 24)	Week16 (week 8 to week 24)	Week 10 (week 2 to week 20)
Reduction in Cicatrising Conjunctivitis Severity Score (median, range)	7.5 (increase 3.5 to reduction 12.5)	7 (increase 3.5 to reduction 12.5)	8 (reduction 0.5 to reduction 12)
Change in visual acuity (median, range)	Nil (loss 8 lines to gain 2 lines)	Nil (loss 8 lines to gain 1 line)	Nil (no change to gain 2 lines)

Table 3.6 Characteristics of patients who developed fibrosis

	Progression of fibrosis (n=4)	No progression (n=15)
Received IVMP	50% (2/4)	47% (7/15)
Age (median, range)	69 (58 - 83)	60 (51 - 82)
Immunopathology positive	100% (4/4)	80% (12/15)
Extraocular Disease	50% (2/4)	47% (7/15)
Autoimmune Disease	0	13% (2/15)
Disease Duration (years) (median, range)	0.6 (0 - 5)	2 (0.1 - 18)
Any Lid surgery	50% (2/4)	67% (10/15)
Topical Steroids used during Trial	100% (4/4)	73% (11/15)
Initial Bulbar Inflammation (median, range)	12 (11 - 16)	11 (8.5 - 14)
Initial Cic Conj Severity Score (median, range)	35 (23.5 - 43)	28.5 (15 - 39)
Fornix Depth- upper (mm) (median, range)	9 (3.5 - 12)	12 (4 - 21)
Fornix Depth- lower (mm) (median, range)	3.5 (1 - 5)	4 (0 - 10)
Median time to control (weeks) and range	18 (8 - 52)	20 (2 - 48)

3.4.5 Adverse effects

With regard to adverse effects, IVMP was associated with transient increased blood sugar levels requiring adjustment of or additional hypoglycaemic medication in 40% (4/10), increased intraocular pressure and bone loss in one patient who had other osteoporosis risk factors including a slim build and lack of weight bearing exercise. No patients developed avascular hip necrosis. Weight gain was more frequent in the control group (50%, 5/10) than in the IVMP group (20%, 2/10) even though both groups of patients received similar 12 week courses of tapering oral corticosteroids (**Table 3.7**).

Major adverse effects occurred in 2 patients in the IVMP group: 1 patient developed haemorrhagic cystitis when cyclophosphamide was withdrawn due to leukopenia and hepatotoxicity, another developed bacterial pneumonia and heart failure. Major adverse effects occurred in 2 patients in the control group: 1 patient developed pneumocystis pneumonia and disseminated cytomegalovirus infection, and another patient developed bacterial pneumonia.

Cyclophosphamide was discontinued in 40% of patients (8/20) before the end of the preferred 12 months duration of therapy because of adverse effects, the most common reasons being lymphopenia/ leukopenia with or without associated secondary infection, hepatotoxicity, anorexia and lethargy.

Table 3.7 Adverse effects

		IVMP (n=10)	Control (n=10)	Total (n=20)
MAJOR ADVERSE EFFECTS	Pneumocystis pneumonia and disseminated CMV		1	1
	Bacterial pneumonia	1	1	2
	Haemorrhagic cystitis following cessation of cyclophosphamide	1		1
STEROID	Increased blood sugar levels	40% (4/10)	10% (1/10)	25% (5/20)
	Weight gain	20% (2/10)	50% (5/10)	35% (7/20)
	Sweating	10% (1/10)	10% (1/10)	10% (2/20)
	Increased intraocular pressure	10% (1/10)	0	5% (1/20)
	Increased systemic blood pressure	0	10% (1/10)	5% (1/20)
	Hirstutism	0	10% (1/10)	5% (1/20)
	Bone loss detected on bone scan	10% (1/10)	0	5% (1/20)
CYCLOPHOSPHAMIDE	Lymphopenia	60% (6/10)	50% (5/10)	55% (11/20)
	mild - requiring dose reduction	20% (2/10)	30% (3/10)	25% (5/20)
	severe - requiring cessation	40% (4/10)	20% (2/10)	30% (6/20)
	Lethargy	50% (5/10)	50% (5/10)	50% (10/20)
	Diarrhoea	0	30% (3/10)	15% (3/20)
	Anorexia	10% (1/10)	30% (3/10)	20% (4/20)
	Insomnia	0	20% (2/10)	10% (2/20)
	Increased micturition	10% (1/10)	10% (1/10)	10% (2/20)
	Alopecia	20% (2/10)	10% (1/10)	15% (3/20)
	Metallic taste in mouth	10% (1/10)	10% (1/10)	10% (2/20)
	Liver function test abnormalities	10% (1/10)	0	5% (1/20)
Proportion of patients who stopped cyclophosphamide before 12 months because of adverse effects		40% (4/10)	40% (4/10)	40% (8/20)
No major adverse side effects with corticosteroid treatment		90% (9/10)	100% (10/10)	95% (19/20)
No major adverse side effects with cyclophosphamide treatment		60% (6/10)	60% (6/10)	60% (12/20)

3.5 Discussion

This pilot randomised controlled clinical trial did not detect any difference in the proportion of ocular MMP patients with control of inflammation at 6 weeks, between those that received adjunctive pulse intravenous methylprednisolone (IVMP) treatment, and the control group. This study was powered to detect a very large treatment difference of 70%, that is, given the published success rate of conventional therapy being 20% at 6 weeks, a difference would have been detected if the efficacy of IVMP was 90% at 6 weeks.

The number of patients recruited is small and the numbers who made it to one year's follow-up are even smaller, so that there is considerable uncertainty regarding exact success rates. It is not possible to rule out any treatment effect at this stage; it is also important to note that at baseline, the treatment group did appear worse with regards several prognostic factors. This study does suggest however that if a difference does exist, it is small and very large numbers of patients would be required for a definitive study. For example, if we assume a success rate of 20 % at 6 weeks and believe that the smallest clinically relevant treatment difference to detect at the 5% level is 20%, in order to achieve a study with 85% power, 93 patients per treatment group would be needed. These figures would then need to be inflated to allow for loss to follow-up, which this pilot study suggests might be considerable. In this study, randomisation was employed so the differences at baseline between the study groups are known to have occurred by chance, but this does emphasise the need to consider whether or not to stratify randomisation for a definitive trial.

Whilst the majority of the side effects of IVMP in this study were minor, transient or manageable with additional medications, one patient did have detectable bone loss despite receiving bisphosphonate, calcium and vitamin D therapy. Bone loss in patients treated with IVMP but not receiving bisphosphonates or oestrogen has

previously been reported (Haugeberg *et al.*, 2004). Given these findings, the use of adjunctive pulse IVMP to control inflammation in severe ocular MMP cannot currently be recommended.

The short-lived effects of pulse IVMP therapy have previously been observed in rheumatoid arthritis, where despite its effectiveness in achieving rapid, temporary relief of disease flares for up to 6 weeks (Williams *et al.*, 1982), repeated monthly pulses over 12 months did not enhance or accelerate the effect of disease modifying drugs such as penicillamine and azathioprine (Hansen *et al.*, 1990). Similarly in systemic lupus erythematosus, the additive effect of pulse IVMP on a background of conventional oral corticosteroid treatment showed an improvement in the first 2 weeks after IVMP administration, but this difference was not maintained at one month or subsequently (Mackworth-Young *et al.*, 1988). Moreover, equivalent doses of oral prednisolone have similar clinical and immunological effects to IVMP in rheumatoid arthritis, indicating that pulse oral treatment can be administered without the inconvenience, cost and potential dangers of intravenous administration (Smith *et al.*, 1988).

Compared to previous studies, the study in this chapter found lower success rates of control of inflammation at 6 weeks, and at other time points during the 12 month follow-up period, with conventional oral corticosteroid and cyclophosphamide. Elder et al (Elder *et al.*, 1995) evaluated 10 ocular MMP patients (19 eyes) in a retrospective study with similarly severe (grade 3 or 4) conjunctival inflammation, and found 5/19 eyes (26%) or 3/10 patients (30%) took 6 weeks or less for inflammation to resolve. It was not specified in Elder's study whether the inflammation recurred, once it had apparently resolved in any eye, but if it is assumed that there was no recurrence of inflammation then by 6 months 15/19 (79%) and 7/10 (70%) of patients had control of inflammation. In contrast, we found 20% (2/10) of patients in the conventional treatment group had control of inflammation at the 6 week time-point, and only 3/8 (37.5%) at the 6 month time-

point. These differences may be partly due to our evaluations occurring at a specific time-point rather than describing the time taken for inflammation to resolve; with the former method, if inflammation has rebounded after initial control, then these patients would subsequently be considered to be uncontrolled at the later time-point of assessment. We found that inflammation rebounded after initial control, in approximately half the patients over the 12 month follow-up period (see **Table 3.3** and **Figure 3.5**). The differences may also partly be due to our definition of control of inflammation being a total bulbar inflammation grade of 6 or less out of a potential maximum total of 16, as assessed by a masked observer. In the Elder study the observer was not masked, and the “grade 1” conjunctival inflammation which was accepted as a successful outcome was an overall grade, rather than specifying a grade for each quadrant of bulbar conjunctiva. Foster reported successful control of inflammation by week 6 in 6/12 (50%) of patients receiving oral prednisone and cyclophosphamide (Foster, 1986), but the pre-treatment conjunctival inflammation in these patients may not have been as severe as those in our study. These studies did not describe the need for additional therapy in combination with cyclophosphamide and oral corticosteroids. In our study, additional therapy (dapsone and /or rituximab) was required after week 12 due to poor inflammatory control in 33.3% (3/9) and 30% (3/10) of the patients in the IVMP and control groups respectively.

We evaluated bulbar conjunctival inflammation by two methods: masked photographic grading and masked observer grading. The masked photographic scores tended to be higher than the masked observer scores, with larger differences observed where there was greater inflammation. This difference could have been due to inconstancies in exposure and lighting on the photographs influencing the photographic grading, as well as the quality of the photographs, given that it was sometimes difficult to clearly photograph all four bulbar conjunctiva quadrants due to the amount of conjunctival tethering and patient discomfort.

In this study, despite all patients receiving maximum tolerated levels of cyclophosphamide immunosuppression, and 40% needing to stop cyclophosphamide because of significant adverse effects associated with dose-related toxicity, progression of conjunctival fibrosis (a decrease in fornix depth and/ or new symblephara) was detected in 4/16 (25%) of all patients reaching the 12 months follow up. This suggests that systemic immunosuppressive therapy alone may not be sufficient to arrest cicatrisation, in part because the high doses required to sufficiently control the inflammation that is driving cicatrisation are not tolerated in this elderly patient population, and in part perhaps because systemic immunosuppression does not specifically control the matrix deposition and remodelling phases of fibrosis.

To the best of the author's knowledge, this is the first pilot clinical trial investigating the possible additive effect of IVMP on rapidity of control of inflammation in patients with severely active ocular MMP commencing conventional oral corticosteroid and cyclophosphamide treatment. However, interpretation of the results is limited by the low power of this pilot study to detect anything other than a very large difference in effect. It is possible that the IVMP group in this study had more severe disease that was difficult to control, indicating selection bias (**Table 3.2**). It would have been ideal to have minimised this selection bias in our randomisation method by stratifying according to severity of the inflammation as well as by study centre. However with such small patient numbers, multiple stratifications are not feasible; this may need to be considered in an appropriately powered trial of treatment for this condition. There was a high exclusion rate in our study, mainly due to the patients declining to participate (18/40 (45%) of eligible patients). This highlights the difficulty of carrying out prospective controlled treatment studies in ocular MMP, where the patients are elderly and reluctant to be involved in the inconvenience of a treatment trial, and the disease is uncommon so achieving the large numbers

necessary for sufficient statistical power to detect smaller treatment differences within a limited time period is difficult without multicentre international efforts.

3.6 Conclusion

Severely active ocular MMP responds slowly to conventional immunosuppressive therapy, and this pilot randomised trial has shown that adjunctive pulse IVMP is unlikely to provide any additional effect, although to make a definitive statement about this would require a trial that is too large (more than 90 patients per group) to be justified by these pilot data. Better treatments that act rapidly to extinguish the autoimmune inflammatory drive are needed, with the intention of suppressing the cicatrisation pathway that is initiated by acute inflammation. Biological treatments such as tumour necrosis factor-alpha antagonists or rituximab are attractive candidate therapies, and require further investigation. The results of this trial will be of substantial value in the design and planning of studies using these biological therapies.

Chapter 4

Tumour necrosis factor-alpha (TNFα) expression in ocular mucous membrane pemphigoid and its effects on conjunctival fibroblasts

4.1 Introduction

As shown in Chapter 3, severely active ocular MMP responds slowly to conventional immunosuppressive therapy, and progression of cicatrisation is observed despite giving maximally tolerated treatment. Alternative therapies that act more rapidly and maintain a sustained effect may more effectively arrest cicatrisation.

Several case reports have described successful use of TNFα antagonists in MMP patients with recalcitrant disease (Canizares *et al.*, 2006; Heffernan & Bentley, 2006; John *et al.*, 2007; Prey *et al.*, 2007). However, based on current evidence, the scientific rationale for the use of TNFα antagonists in MMP is weak. Although it has been reported that serum levels of TNFα are elevated in MMP compared with normal controls (Lee *et al.*, 1993), there are limited studies examining TNFα expression in MMP tissue. Tissue expression may be important because in rheumatoid arthritis, whilst there is no evidence that plasma TNFα levels can predict the clinical response to TNFα antagonists, synovial expression of TNFα appears to be a significant predictor of response to TNFα antagonists (Wijbrandts *et al.*, 2007).

Given our findings in Chapter 2 of conjunctival fibrosis still progressing despite apparent clinical control of inflammation by conventional immunosuppressive therapy, better understanding of the immunological changes in treated tissue compared with actively inflamed tissue would assist and guide in developing adjunctive local therapies to target conjunctival fibrosis.

There is controversy about whether TNFα is a pro-fibrotic or anti-fibrotic cytokine (Distler *et al.*, 2008; Saika *et al.*, 2006; Sullivan *et al.*, 2005; Theiss *et al.*, 2005); if it has anti-fibrotic effects on conjunctival fibroblasts, antagonist therapy could worsen conjunctival fibrosis. Before large controlled studies of the use of TNFα

antagonists in ocular MMP can be recommended, further investigations are necessary, including confirming its tissue expression in ocular MMP, and establishing whether TNFα has pro-fibrotic or anti-fibrotic effects on human conjunctival fibroblasts.

The pathophysiology of conjunctival fibrosis in ocular MMP is poorly understood. Observations from other chronic mucosal fibrotic disorders provide insights into what could be occurring in ocular MMP. The role of CD40 signalling in regulation of inflammation and fibrosis has been demonstrated in the lung (Kaufman *et al.*, 2001; Sempowski *et al.*, 1997). Chronic stimulation through CD40 may hence lead to fibrosis. Rather than merely acting as structural cells, fibroblasts thus appear to participate actively in the immune response by producing cytokines and chemokines that initiate the recruitment and retention of bone marrow-derived immune effector cells (Flavell *et al.*, 2008). Given that conjunctival fibrosis in ocular MMP can still progress despite apparent clinical control of inflammation by conventional immunosuppressive therapy, better understanding of the cellular and molecular changes in treated tissue compared with actively inflamed tissue would assist and guide in developing adjunctive local therapies to target conjunctival fibrosis.

4.2 Aim

The aim of the study in this chapter was to investigate whether TNFα is expressed in conjunctival MMP tissue, evaluate changes in TNFα expression after systemic immunosuppressive treatment, and to determine whether TNFα has pro-fibrotic or anti-fibrotic effects on normal human conjunctival fibroblasts.

4.3 Research design and methods

4.3.1 Conjunctival Biopsies

Bulbar conjunctival biopsies between 3 x 3mm to 4 x 4mm were obtained from patients with ocular MMP. Topical anaesthesia with proxymetacaine 0.5% or

oxybuprocaine 0.4% was given and biopsies of superior bulbar conjunctiva distant from the fornix were obtained using spring scissors and blunt forceps, as shown in **Figure 1**. Superior bulbar conjunctiva was chosen because healing of the biopsy site would be facilitated by the upper lid providing protection from exposure; the fornix was avoided because biopsies of fornix have been reported to cause further scarring (Mondino *et al.*, 1979). The biopsies were divided into 3 pieces: 1 piece for direct immmunofluorescence (DIF) microscopic diagnosis, 1 piece for immunohistochemistry, and 1 piece for conjunctival fibroblast explant culture. The piece of conjunctiva for DIF was placed directly into Michel's medium, and the other 2 pieces were placed into Leibovitz L-15 medium containing 100IU/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B (all from Gibco Invitrogen Ltd, Paisley, Scotland UK) to support cell growth in non-CO_2-equilibrated conditions during transportation to the laboratory.

The patients were classified according to the grade of ocular inflammation present as having active disease with acute inflammation (**Figure 4.2 A**) (n=10), or chronic disease (n=10) without clinically apparent inflammation (**Figure 4.2 B**), after treatment with immunosuppressive therapy. The diagnosis of ocular MMP was based on clinical presentation and positive immunopathology. However, in the presence of characteristic clinical features, negative immunopathology did not exclude the diagnosis of ocular MMP. Conjunctivae from 10 individuals with normal conjunctiva undergoing routine cataract surgery were used as controls; these biopsies were harvested just before the surgical procedure began, after instillation of topical anaesthesia.

Details of the patients from whom conjunctival biopsies were used for the TNFα experiments presented in this chapter are shown in **Table 4.1.** A larger number of biopsies were taken but only approximately 50% of the biopsies harvested successfully grew conjunctival fibroblasts for culture. The tenets of the Declaration of Helsinki were followed, and approval from the Oxfordshire Research Ethics

Committee (for biopsies of normal conjunctiva and for conjunctival biopsies from ocular MMP patients participating in the clinical trial described in chapter 2; see **Appendix 3**) and from the Moorfields and Whittington Ethics Committee (for biopsies of active and uninflamed conjunctiva from clinic patients with ocular MMP; see **Appendix 5**) was granted. Informed consent was obtained from all patients and normal controls who provided conjunctival tissue for the study.

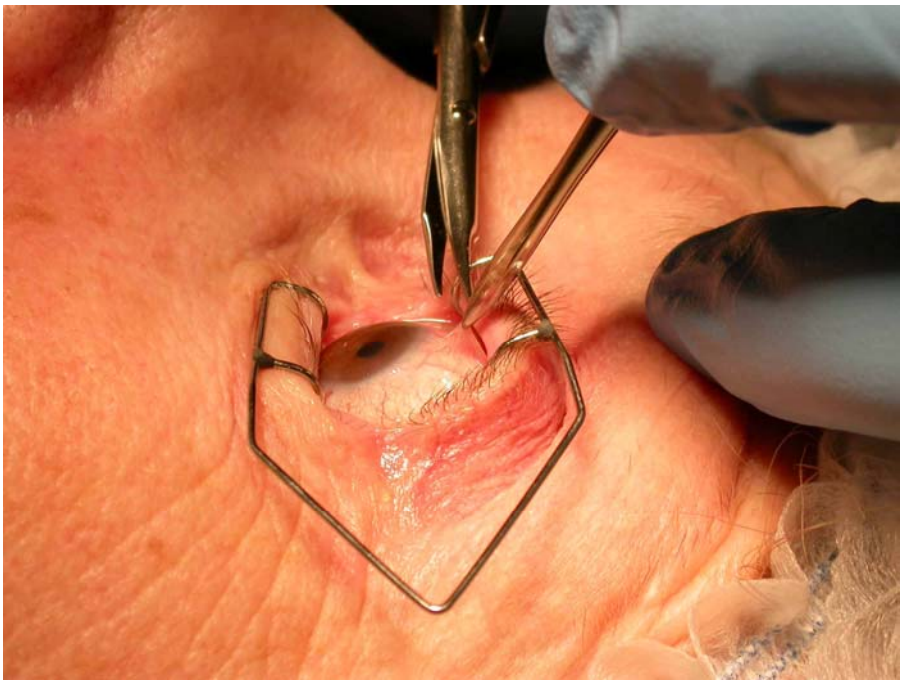


Figure 4.1 Biopsy of superior bulbar conjunctiva

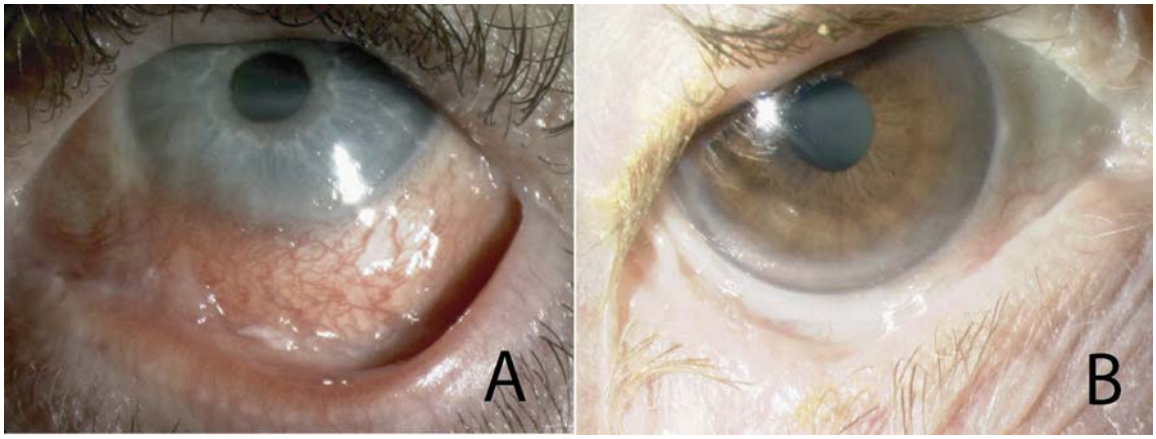


Figure 4.2 A. Patient with actively inflamed ocular mucous membrane pemphigoid (active case 1, L eye), **B. Following immunosuppressive therapy, patient with clinically uninflamed treated ocular mucous membrane pemphigoid** (uninflamed case 8, R eye)

Table 4.1 Details of patients and normal controls whose conjunctival biopsies were used in TNFα studies

Diagnosis	Case number	Age	Gender	Disease duration (yrs)	Bulbar inflammation grade (0 to 4)	Tauber stage (Upper stage/ Lower stage)	Topical therapy	Systemic therapy	Other eye pathology
Active MMP	1	54	F	0.5	3	IIcIIId/ IIcIIId	hypromellose, acetylcycsteine, yellow soft paraffin	nil	
	2	80	M	10	3	IIbIIIc(2)/ IIdIIId(2)	carmellose	mycophenolate + dapsone	
	3	60	F	2	3	I/ IIcIIIc(3)	carmellose	mycophenolate + dapsone	
	4	74	M	2	3.5	IIa/ IIcIIIc(2)	dorzolamide, bimatoprost	nil	glaucoma
	5	76	F	2	2.5	IIc/ IIbIIIa(2)	prednisolone, hypromellose, carmellose	mycophenolate + dapsone	sicca, blepharitis
	6	51	F	0.1	3	I/ IIIa(1)	nil	nil	
	7	57	M	0.5	3	IIb/ IIcIIIb(2)	dexamethasone, lacrilube	nil	
	8	64	F	1	2	IIa/ IIbIIIc(2)	prednisolone	nil	
	9	83	F	7	3	IIa/ IIbIIIb(2)	brimonidine, latanoprost, timolol, dorzolamide, carmellose	mycophenolate + doxycycline	glaucoma
	10	72	F	0.25	4	IIa/ IIcIIIc(2)	ofloxacin, cyclopentolate, carmellose	nil	microbial keratitis
Treated uninflamed MMP	1	59	F	10	1	IIbIIIb(2)/ IIdIIId(2)	carbomer 980	dapsone	
	2	86	F	15	1	IIb/ IIcIIIb(2)	nil	nil	
	3	78	F	10	1	IIbIIIb(2)/ IIdIIId(2)	nil	nil	
	4	84	F	2	0	IIb/ IIcIIIc(2)	nil	mycophenolate	
	5	66	F	2	0	IIb/ IIcIIIb(2)	nil	dapsone	
	6	76	F	4	1	IIc/ IIcIIIc(3)	betaxolol	dapsone	glaucoma
	7	59	M	4	1	I/ IIa	hypromellose, carmellose, liquid paraffin, chloramphenicol	cyclophosphamide	
	8	76	M	6	1.5	IIdIIId(2)/ IIbIIIb(2)	chloramphenicol, hypromellose, retinoic acid, acetylcysteine	cyclophosphamide	blepharitis
	9	60	F	3	1	I/IIaIIIa(1)	carmellose	cyclophosphamide + dapsone	
	10	62	F	0.75	0.5	IIa/ IIcIIIa(1)	hyaluronate	prednisolone	
Normal control	1	50	F	-	0	-	nil	nil	cataract
	2	76	M	-	0	-	nil	nil	cataract
	3	65	M	-	0	-	nil	nil	cataract
	4	70	F	-	0	-	nil	nil	cataract
	5	65	M	-	0	-	nil	nil	cataract
	6	73	M	-	0	-	nil	nil	cataract
	7	84	F	-	0	-	nil	nil	cataract
	8	57	M	-	0	-	nil	nil	cataract
	9	84	F	-	0	-	nil	nil	cataract
	10	62	M	-	0	-	nil	nil	cataract

MMP= mucous membrane pemphigoid

4.3.2 Immunohistochemistry

Expression of TNFα protein was investigated by immunohistochemistry on glycol methacrylate (GMA) resin-embedded sections of conjunctiva, prepared as described previously (Bernauer *et al.*, 1993b). GMA-embedded sections generate an immunophenotypic profile similar to that obtained in frozen sections, while yielding far superior morphology and greater numbers of sections from small biopsies (Britten *et al.*, 1993). Fresh biopsy tissue was immediately placed in ice-cooled acetone containing protease inhibitors, stored overnight at -20^{o}C, then processed for, and embedded in, glycol methacrylate (GMA) resin using the JB4 kit (TAAB Laboratories, Aldermaston, UK). The blocks were stored at -70^{o}C. An ultramicrotome was used to cut 2μm sections, mounted on poly-L-lysine-coated slides and air dried.

Endogenous peroxidase was inhibited using a solution of 0.3% hydrogen peroxide in 0.1% sodium azide for 10 minutes (Sigma-Aldrich, Dorset, UK), followed by 2 five-minute PBS (phosphate-buffered saline) washes then incubation for 30 minutes with 10% foetal calf serum to prevent non-specific binding of monoclonal antibody. The sections were incubated overnight at room temperature with a mouse antibody against human TNFα (Abcam Ltd, Cambridgeshire UK [ab9579]) at 1:50 dilution. After washing in PBS, biotinylated rabbit anti-mouse immunoglobulin 1:200 (Dako, Cambridgeshire UK) was applied to the sections for 2 hours. Following a further wash in PBS, the slides were incubated with streptavidin-peroxidase 1:300 (Dako) for 2 hours, again washed in PBS and finally developed for 25 minutes with amino-ethyl carbazole (AEC, Dako), forming a red AEC reaction product. For TNFα-CD3 double staining, the slides were washed with PBS and the immunohistochemistry process was repeated using a primary mouse antibody against human CD3 (Dako) at 1:10 dilution, biotinylated anti-mouse antibody (Dako) as the secondary antibody, and 3,3'diaminobenzidine (DAB) instead of AEC as chromagen. This formed a brown DAB reaction product.

The specimens were counterstained with Meyer's hematoxylin (Dako) and mounted with glycergel (Dako). Double staining was identified by a combined red-black colour, due to the combination of a positive reaction to AEC and DAB. Human tonsil sections were used as positive controls, and the two negative controls used were substituting the primary antibody for foetal calf serum or an isotype matched, irrelevant monoclonal antibody (Dako). The number of cells stained in the conjunctival stroma were counted in a masked fashion in at least 5 representative high power fields per patient, using an Olympus BX51 microscope with image analysis software. The presence of positive stromal or epithelial staining was also graded on a scale of 0 to ++++, based on both the number of cells stained and staining intensity.

The presence of protein expression by immunohistochemistry was considered to be sufficient evidence, given time constraints and limited amounts of tissue available, of TNFα being involved in the immunopathology of ocular MMP. Immunohistochemistry was favoured because double staining could give additional information about the cellular source of TNFα expression, whereas alternative methods such as Western blotting would involve utilizing the whole specimen to confirm protein expression, but could not give additional information regarding the cellular source. Once the staining method had been optimized including dilutions, all the immunohistochemical staining was carried out on the active (n=10), uninflamed (n=10) and control (n=10) sections at one sitting, to minimize variations in staining technique.

4.3.3 Isolation of conjunctival fibroblasts

Explant culture

Fibroblasts were grown as explants from the conjunctival biopsies as previously described (Khaw *et al.*, 1992). Using aseptic technique in a laminar flow hood, the piece of conjunctiva was placed in Dispase II (Roche Diagnostics, Basel, Switzerland) for 1 minute then scraped gently with a scalpel to remove the

epithelium. The tissue was placed in a collagen-coated well of a 24-well tissue culture plate (Fisher Scientific, Leicestershire UK), then a 10mm diameter glass cover slip (Raymond A Lamb, East Sussex UK) was placed on top, and the biopsy under the coverslip in the culture well was incubated at 37^{o}C for 30-45 minutes depending on the size and moistness of the biopsy (shorter duration for smaller, less moist biopsies, to avoid drying out and death of the cells), before gently adding 80µl of fibroblast culture medium (FCM, see below) to the well. The purpose of the coverslip and short period of initial incubation without cell culture medium were to promote tissue fixation and adhesion to the tissue culture surface, which is necessary to facilitate fibroblast outgrowth, prior to adding culture medium which would otherwise dislodge the tiny piece of tissue from the tissue culture surface. The coverslip also protected the small biopsy from potential displacement and loss when refeeding during early growth. After the first 24hours, when adhesion was evident, more culture medium was gently added so that there was at least 500µl medium in the well. The surrounding wells were filled with sterile PBS to ensure that the biopsy remained in a humid environment.

The collagen-coated wells were prepared prior, using sterile 0.1mg/ml rat tail type I collagen mixture, prepared by diluting stock 5mg/ml collagen mixture (see section **4.1.1** below for preparation of 5mg/ml collagen mixture) 1:50 using 1:1000 acetic acid solution, and incubating 500µl of this diluted collagen mixture per well of a 24-well plate for 2 hours at 37^{o}C, then removing the collagen and washing the well 4 times with sterile PBS to minimize cell toxicity due to the acetic acid contained in the collagen. These plates were then dried out under the flow hood, sealed with parafilm and stored at 4^{o}C.

Feeding biopsies and harvesting passage 1 cells

Fibroblast culture medium (FCM) was composed of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated foetal calf serum, 100IU/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B (all from

Gibco) and the biopsies and cells were cultured at 37^{o}C with 5% (v/v) CO_2 in air. The primary biopsies were fed once weekly, and fibroblasts were usually noted within 1-3 weeks of culture. Any residual epithelial cells appeared to die and were not evident beyond 4 weeks. Once the well was densely confluent at 4 to 8 weeks, the cells peripheral to the biopsy were displaced from the culture surface by aspirating the culture medium, washing twice with warm sterile PBS, followed by incubation with 400µl of prewarmed 1 x trypsin/ EDTA (Gibco) for 1-2 minutes, and then gentle pipetting and aspiration of the displaced cells peripheral to the biopsy, whilst avoiding dislodgement of the primary biopsy. The PBS washes were necessary to wash the serum off and allow the trypsin to work. The aspirated cell suspension was then placed into 6-well culture plates containing 2 ml FCM. It was necessary to subculture the passage 1 cells into 6 well plates as a first stage, because there were insufficient passage 1 cells to support growth in 25cm^2 flasks.

Passaging cell cultures in plates and flasks

Once the 6 well plates were confluent, cells were then passaged 1:3 with trypsin/EDTA, either in 6 well plates or in 25cm^2 to 175cm^2 cell culture flasks (Nunc, VWR International, Leicestershire, UK). Cells were assessed weekly by viewing with a phase contrast inverted microscope and passaged cells were fed once or twice weekly. Cells were passaged by aspirating the culture medium, washing twice with warm sterile PBS, incubating for 1-2 minutes with warm trypsin/ETDA then gently pipetting to mechanically dislodge the cells from the plates, or tapping the undersurface of the flasks. The plates or flasks were then viewed at x100 magnification with a phase contrast inverted microscope (Nikon). When most of the cells were seen to be rounded up, the action of trypsin was stopped by adding an equivalent volume of FCM containing 10% foetal calf serum. This cell suspension was then centrifuged for 5 minutes at 1000 rpm and the supernatant aspirated to obtain a cell pellet which was then resuspended in culture medium as necessary for passaging, or for experiments. Unless otherwise specified, cultures were used between passages 3 and 7 for experiments, and

cultures were assessed for typical fibroblast morphology by phase contrast microscopy before every experiment. By passage 3 onwards, contaminating epithelial cells had been eliminated in cultures that had been fed only FCM, due to the fastidious nutritional requirements of epithelial cells.

Storage and recovery of cells in liquid nitrogen

Additional cells were cryopreserved by resuspending the cell pellet in FCM which contained 20% foetal calf serum, then adding drop-wise an equivalent volume of 20% dimethylsulfoxide (DMSO) solution (prepared by adding 2ml DMSO to 8ml 20% serum-containing FCM).This cell suspension was then distributed into 1ml cryovials with approximately 1 x 10^6 cells/ml per vial, and the cryovials were placed into the inner chamber of a plastic container containing isopropanol in the outer chamber, which was designed to slowly reduce the temperature in the inner chamber by 1^oC increments to avoid cell toxicity. This container was placed in the -70^oC freezer overnight, then the cryovials were transferred to liquid nitrogen storage (> -180^oC). Cryopreserved cells were recovered by gently defrosting the mixture in the cryovial using a 37^oC waterbath, then immediately adding the liquid mixture, drop-wise, to 10ml warmed FCM containing 20% foetal calf serum. This cell suspension was then centrifuged at 1000rpm for 5 minutes, and the cell pellet was resuspended in FCM for seeding into an appropriate sized flask or plates. The use of 20% serum-containing medium was found to protect the cells from DMSO toxicity.

Serum-free medium (SFM)

Serum-free medium (SFM), composed of Dulbecco's modified Eagle's medium (DMEM) and 0.1% bovine serum albumin (Sigma-Aldrich), supplemented with 100IU/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin B was used wherever possible in experiments where the effect of the addition of a cytokine was being evaluated. Testing under serum-free conditions eliminated the possibility that the observed response could be due to the activity of unspecified

cytokines and growth factors present in serum-containing medium, even with the use of heat-inactivated serum.

The cultured conjunctival fibroblasts were used in experiments assessing several aspects of fibroblast activity, including migration, extracellular matrix contraction, matrix metalloproteinase production and surface expression of markers of activation and co-stimulatory molecules.

The optimal concentration of cytokine (e.g. TNFα) used in experiments was initially determined from previous literature (Leonardi *et al.*, 2003). If this level did not result in a significant response, a range of concentrations of TNFα [1, 10, 100ng/ml] was then evaluated in a preliminary dose-response experiment. The concentration that showed the greatest response in this preliminary experiment was then selected as the optimal concentration for that assay. 3T3 cells (a mouse fibroblast cell line) were used for initial calibration experiments that compared a serum free negative control to a FCM positive control.

4.3.4 Fibroblast migration

Cell culture inserts incorporating polyethylene terephthalate membranes with a pore size of 8μm (BD Falcon, VWR International, Leicestershire UK), which fit into wells in a tissue culture plate, were used to assess fibroblast migration in the presence of TNFα. Cells were seeded into the inserts at a density of 8000 per insert in 200μl SFM and allowed to attach to the upper surface of the membrane for 4 hours. A volume of 700μl of the medium being tested for chemoattractant properties was added to the well in the tissue culture plate, so that the test medium was in contact with the undersurface of the membrane in the culture insert. To investigate the effect of TNFα on normal conjunctival fibroblast migration, 10ng/ml of recombinant human TNFα (R&D Systems Europe Ltd, Abingdon UK) in SFM was added to the culture well. SFM alone was used as a negative control, and 10% serum-containing FCM as a positive control. The cells were incubated for 16 hours

to permit migration through the pores, to the undersurface of the membrane. The culture inserts were then washed with PBS to remove excess protein, fixed in 70% (v/v) methanol for 5 minutes, then stained with Mayer's haematoxylin (Dako) for 30 minutes and rinsed in tap water. Settled cells on the upper surface of the membrane in the culture inserts were removed using cotton buds. The number of migrated cells on the undersurface of the membrane was counted per 10x objective field (average of 5 fields) by taking photographs on an inverted phase contrast microscope and using cell counting software (Image J public domain Java image processing program http://rsb.info.nih.gov/ij/).

4.3.5 Collagen contraction model

To assess matrix contraction by conjunctival fibroblasts, fibroblast-populated collagen lattice models were used, similar to those described by Mazure and Grierson (Mazure & Grierson, 1992). The collagen gel contraction model was selected as an experimental method for these studies in part because it is more physiological, as the cells acquire tissue-like phenotypic characteristics not otherwise observed when growing cells in monolayers on plastic (Elsdale & Bard, 1972), and in part because it maximized use of the limited numbers of primary conjunctival fibroblasts able to be obtained from the small conjunctival biopsies, in that it enabled use of one type of experiment to potentially assess 3 aspects of fibroblast function: (1) collagen matrix contraction, (2) secretion of matrix metalloproteinases, collagen and other molecules of interest in the conditioned medium, and (3) expression of surface or intracellular molecules on the fibroblasts in the collagen matrix by immunostaining and confocal microscopy.

Preparation of fibroblast-populated collagen gels

To prepare the collagen gels, a 5mg/ml collagen gel mixture was first made up by adding 1:1000 acetic acid solution (50µl glacial acetic acid in 50ml distilled water, filter sterilized) to 100mg of rat tail type I collagen (C-8897, Sigma-Aldrich) to give a total volume of 20ml. This mixture was incubated at 37^{o}C for 1-2 hours to dissolve,

then kept at 4^{o}C. The collagen gels were prepared by adding 0.6ml of the collagen gel mixture to 0.35ml concentrated medium stock solution (15ml of 10X DMEM (Gibco), 35ml sterile distilled water, 1.5ml of 10,000U/ml penicillin/10,000µg/ml streptomycin solution, 1.5ml of 2mM glutamine, and 4ml of 7.5% (w/v) sodium bicarbonate), which would provide enough mixture for 6 gels in a 48 well plate. The pH of this mixture was adjusted to 7.4 by adding 1M NaOH dropwise until the solution changed colour to pink. Then 0.15ml of the appropriate number of fibroblasts (see below) suspended in concentrated medium was added to the collagen mixture, and 150µl of this cellular gel mixture was quickly cast into each of 6 wells of a 48 well plate. The plate was incubated at 37^{o}C for 2 hours to polymerise the collagen lattices.

The gels were then fed with 0.5ml of the test culture medium (either SFM, SFM containing 10ng/ml TNFα, or 10% gelatinase-free serum-containing FCM) just prior to detaching the gels from the culture wells. This was done by using a micropipette to gently release the gel's anchorage at 4 points around its circumference, whilst avoiding tearing the gels, then checking for free flotation of the gels using a gentle circular rotating motion.

The gels were digitally photographed at days 1, 3 and 7, and the gel areas were calculated using image analysis software (Image J). Conditioned media from the collagen lattice experiments was collected between days 0-3 and 3-7, or days 0-7, to assess matrix metalloproteinase secretion. At the end of the experiment, the collagen gels were washed with PBS and fixed with 4% paraformaldehyde (which was diluted to 0.4% paraformaldehyde after 24hrs) in order to preserve them for immunostaining and confocal microscopy at a later date.

Gelatinase-free serum was produced by incubating foetal calf serum with gelatin sepharose beads (Gelatin Sepharose 4B, Amersham Biosciences, Buckinghamshire UK) at a concentration of 10% with gentle rocking for 2 hours.

This mixture was centrifuged at 4000rpm for 10 minutes to separate the beads from the gelatinase-free serum.

Free-floating relaxed versus attached stressed collagen gel models

In these free-floating relaxed collagen gels, contraction of collagen by the fibroblasts occurs in the absence of an external mechanical load. Alternative culture models also exist (Grinnell, 2000), where the gel matrix remains attached to the culture well (attached gel model), or where the collagen gel remains attached to the well for 48 hours before release (stress-relaxation gel model) (see Figure **4.3**). When the gel remains attached to the culture well, an external mechanical load develops during contraction of collagen by the fibroblasts, and assembly of actin stress fibres occurs within the cells, indicating differentiation into myofibroblasts (Garrett *et al.*, 2004). This method was used in experiments which will be described in **Chapter 6**.

Determining the optimum concentration of fibroblasts

To determine the minimum number of human conjunctival fibroblasts required to give a measurable contraction of the collagen lattice over a period of 7 days, preliminary experiments were conducted, seeding lattices with fibroblasts at 5 x 10^4, 1 x 10^5 and 2.5 x 10^5 cells per 0.15ml concentrated medium. It was found that 2.5 x 10^5 cells per 0.15ml concentrated medium yielded the required degree of contraction, when FCM was used as the test culture medium. This number of cells was therefore used for all subsequent experiments.

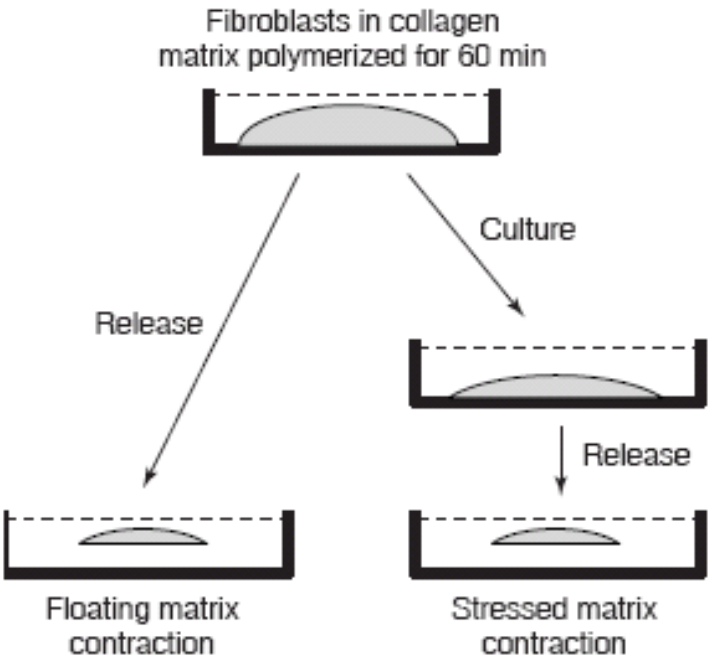


Figure 4.3 Models for the contraction of free-floating relaxed and stressed matrices. In the free-floating relaxed model, the cell-embedded matrix is released from the culture well immediately after matrix polymerisation. Polymerisation can be carried out for 1 or 2 hours. In the stress-relaxation model, the cell-embedded, polymerised matrix is cultured attached to the well, during which force exerted by the cells results in the development of isometric tension. Following this, the matrix is released (relaxed) to initiate contraction. Adapted from Grinnell F, 2000.

4.3.6 Matrix metalloproteinase protein secretion

Conditioned media collected from the collagen lattice experiments were analysed for protein expression levels of matrix metalloproteinase (mmp)-1, mmp-2, mmp-3, mmp-8, mmp-9, mmp-10, mmp-13 and tissue inhibitor of matrix metalloproteinase (timp)-1, timp-2 and timp-4 by using an antibody-coated membrane array kit (Raybiotech Inc, Norcross, GA, USA) in accordance with the manufacturer's instructions. This antibody array uses a biotin-conjugated primary antibody to detect the mmp/ timp protein bound to arrayed anti-mmp/ anti-timp antibody on the membrane, horse radish peroxidase (HRP)-labelled streptavidin is then used to detect the biotin, the generation of light by chemiluminescent substrates of the

enzyme HRP is detected by exposure of the membrane array to X-ray film, and the signal intensities are quantified by densitometry. Mmp and timp levels in 10% gelatinase-free serum-containing FCM and SFM medium were subtracted from the appropriate conditioned medium results. The array detects both the pro- and active forms of mmps and timps.

Reasons for selecting this antibody array method rather than gelatin zymography or ELISA (enzyme-linked immunosorbent assay) included: (1) it was more efficient in terms of both the time taken to acquire results, and the use of a limited volume of samples, given that it was able to detect 10 molecules simultaneously from 1ml of conditioned medium, (2) it is reported to have a greater sensitivity and greater detection range than ELISA, and (3) although it was only able to provide relative expression levels, rather than actual pg/ml concentrations as an ELISA would, this quantitation was more accurate than quantitation of zymography results, which relies on detecting differences in shades of light or dark from photographs. Before commencing the antibody array experiments, gelatin zymography was carried out on one occasion to confirm the presence of gelatinolytic activity in a selection of conditioned medium samples (data not shown).

4.3.7 Fibroblast proliferation

Fibroblast cell division was assessed by flow cytometric measurement of dilution of the fluorescent label CFSE (carboxyfluorescein diacetate, succinimidyl ester) (Vybrant® CFDA SE Cell Tracer Kit, Invitrogen, Paisley UK), bound to proteins in the cell. For each division, the amount of CFSE in each daughter cell is half that of the parent. This technique was selected after multiple unsuccessful experiments using alamar blue indicator dye to detect cell proliferation, where positive and negative control conditions did not give reproducible or logical results.

Flow cytometric assessment of CFSE dye dilution offers advantages in terms of providing much more information about the kinetics of cell proliferation, given that it

permits visualization of eight to 10 discrete cycles of cell division, and can track proliferation in minor subsets of cells and follow the acquisition of differentiation markers or internal proteins linked to cell division (Lyons, 2000). A typical graph showing CFSE tracking of cell division is depicted in **Figure 4.4**. This technique also allows conjugated antibodies to be used to probe surface marker changes as cells divide, or changes in expression of internal molecules such as cytokines. An additional advantage of the technique is the ability to recover viable cells which have undergone defined numbers of cell divisions by flow cytometric sorting, allowing functional studies to be performed. In comparison, other commonly used assays of cell proliferation give only limited information, as they usually measure division at a population level (Wallace *et al.*, 2008). There were initial plans to simultaneously assess cell apoptosis during these cell division experiments, by labeling the cells with a marker such as Annexin V, but this work was not able to be carried out due to time constraints.

Preliminary CFSE experiments were conducted using a fibroblast cell line (3T3) to determine the optimum cell density to be plated, the optimum incubation period for detection of maximal cell division, and the optimal CFSE concentration. Initially the effect of adding cytokine under serum-free conditions was attempted, but the cells did not survive these conditions so all further experiments were carried out using 10% serum-containing FCM.

CFSE cell division assay method

Confluent fibroblasts were cultured in serum-free medium for 24 hours prior to commencing the experiment, in order to synergise their cell cycles. The cells were detached from the flasks with trypsin/EDTA and the cell pellet was washed once with SFM. The volume of the cell pellet was assessed (eg 200µl). The CFSE stock was made up prior by adding 90µl DMSO (dimethylsulfoxide) to Component A, and aliquoted into 7µl units. This CFSE stock was then diluted 1:100 with SFM and 1µl of this solution was incubated per 200µl cell pellet at 37^{o}C in the waterbath for 10

minutes, then 1ml ice cold FCM was added to stop the reaction. This suspension was centrifuged and the cell pellet was resuspended in 1ml ice cold FCM for 30 minutes at room temperature, to allow free CFSE to exit the cells. The final cell pellet was resuspended in FCM at 2 x 10^5 cells/ml. 1:4 of these CFSE-containing cells were immediately acquired for flow cytometry (time 0) (10,000 events; Facscalibur, BD Pharmigen). The remaining cells were seeded into 6-well culture plates at a density of 4 x 10^5 cells per well in FCM for 72 hours (time 72h). The effect of TNFα [10ng/ml] in FCM on cell division was compared to FCM alone. When there were sufficient cells, the wells were set up in duplicate or triplicate. After 72 hours, the cells were detached with trypsin/EDTA and at least 10,000 events were acquired in the live region (Facscalibur, BD Pharmigen). Winlist software (Verity Software House, Topsham, ME, USA) was used to assess the ratio of divided cells, which was calculated by measuring the number of cells with a diluted CFSE concentration per 5000 undivided cells.

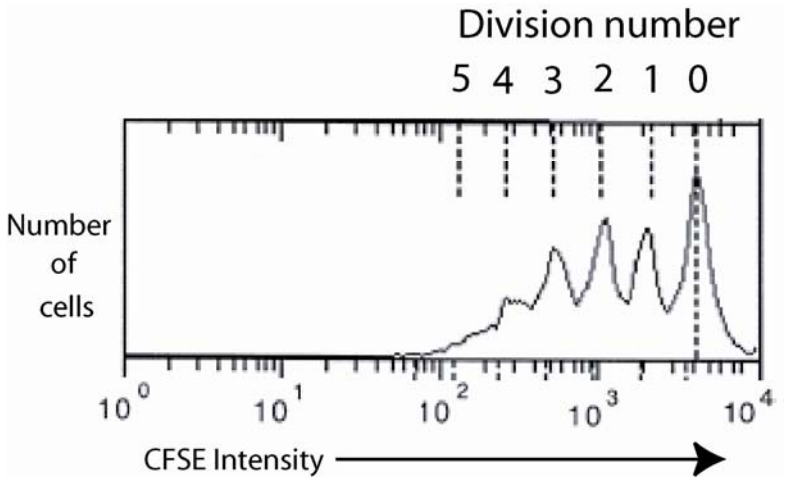


Figure 4.4 Representative plot showing CFSE tracking of cell division. B cells labelled with CFSE were harvested and analysed by flow cytometry after 3 days in culture. Cell division is characterised by sequential halving of CFSE fluorescence, generating equally spaced peaks on a logarithmic scale. Dashed lines indicate the division cycle number. Note the slow decay in intensity of peaks, independent of division. Adapted from Lyons AB, 2000.

4.3.8 Immunofluorescence staining and flow cytometry

Expression of markers of activation (HLA-DR, ICAM) and costimulatory molecules (CD80, CD86, CD40, CD40 ligand) on conjunctival fibroblasts in response to stimulation by TNFα was evaluated by flow cytometry. Confluent fibroblasts were cultured in 6 well plates in the presence of various concentrations of TNFα [1, 10, 100ng/ml] or IFNγ [1ng/ml] (Peprotech, London UK) in SFM, for 72 hours. A range of TNFα concentrations was used for this assay because there was no previous data in the literature. If a response was observed, testing a range of concentrations could also indicate whether there was a dose-dependent effect. IFNγ was tested in parallel as a positive control, given that upregulated CD40 expression in response to IFNγ has previously been reported (Kaufman *et al.*, 2001). For three-colour immunofluorescence staining, fibroblasts were washed with Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (HBSS) (Gibco) and detached with collagenase [1mg/ml] (Sigma-Aldrich) in HBSS. The cell suspension, containing at least 1 x 10^5 cells, was centrifuged at 1000rpm for 5 minutes, the cell pellet was resuspended in 10% mouse serum for 20 minutes to block non-specific binding, then the cells were stained with 5µl each of either anti-ICAM-1^{PE}, anti-$CD80^{FITC}$and anti-$HLA\text{-}DR^{PerCP}$, or anti-$CD154^{FITC}$, anti-$CD86^{PE}$, and anti-$CD40^{PECy5}$ for 30 minutes at 4^oC, before washing twice and acquiring 10,000 events for flow cytometry (Facscalibur, BD Pharmigen). Isotype matched, irrelevant monoclonal antibodies were used as negative controls. Winlist software (Verity Software House, Topsham, ME, USA) was used for analysis. Analyses were performed on a population of live cells gated by forward and side scatter to include the fibroblast population. At least 10,000 events were acquired in the live region, and the percentages of positive cells were calculated after subtracting background staining. Mean fluorescence intensity was used to measure of intensity of expression.

Excluding contamination by epithelial cells

To be confident that the cells analysed in these flow cytometry experiments were fibroblasts alone, without any contaminating epithelial cells, cultures selected for these flow cytometry experiments were at least passage 4 or 5, had typical fibroblast morphology, and were unlikely to contain any epithelial cells by this time given that epithelial cells are more fastidious, requiring additional agents such as epidermal growth factor, cholera toxin and insulin in culture medium to ensure their survival.

Investigating fibroblast and epithelial cell markers

It was planned initially that flow cytometry experiments would be carried out on early passage (passage 0 or 1) cells to detect differences in activation marker expression between cells derived from ocular MMP tissue compared with normal conjunctiva. It was unknown whether the phenotype of the ocular MMP cells would be retained after passaging in tissue culture, and it was possible that the phenotype would revert to a "common" *in vitro* conjunctival fibroblast phenotype once the unique tissue environment had been removed, so evaluating the cellular phenotype of passage 0 or 1 cells was considered important. Given that contaminating epithelial cells were likely to be present in passage 0 or passage 1 cell populations, and we wanted to be able to separately identify the fibroblasts from epithelial cells on flow cytometry, preliminary experiments were conducted to evaluate candidate fibroblast markers such as FSP1 (fibroblast surface protein-1), Thy-1 (CD90) and epithelial markers such as CD326, pan-cytokeratin, and CD44 (see **Appendix 6**). Due to time constraints, these experiments were not repeated so the preliminary results could not be fully assessed, and no conclusions were able to be made.

4.3.9 Statistical Analysis

Differences between groups were examined for statistical significance using the Mann-Whitney U test, or one-way analysis of variance (ANOVA). Students' t-test

was used when comparing means within each sample. Linear regression was used to analyse for a significantly non-zero slope relationship between TNFα concentration and intensity of ICAM expression. $P < 0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 Expression of TNFα in ocular MMP conjunctival tissue

Expression of TNFα in the substantia propria of normal control conjunctival sections was confined to intravascular spaces, with very little expression in the stromal tissues, with sections from only 3 of the 10 normal patients (30%) showing occasional positive stromal cells (**Figure 4.5 A**). In contrast, in active ocular MMP there were many stromal cells expressing TNFα (234 ± 94cells/mm^2) (**Figure 4.5 B**), 32% (76/234) of which were also positive for the T cell marker CD3 (**Figure 4.6 A**). It is likely that the remaining TNFα+CD3- cells are monocytes/ macrophages, because they are, after lymphocytes, the second most abundant stromal inflammatory cells found in ocular MMP (Bernauer *et al.*, 1993b; Rice & Foster, 1990; Sacks *et al.*, 1989).

Following immunosuppressive therapy, in clinically uninflamed ocular MMP, the number of stromal TNFα-expressing cells was significantly reduced to 38% of the number of cells present in active disease (90 ± 46 cells/mm^2, $P < 0.05$), but this was still 9-fold greater than the number of stromal cells present in normal controls (10 ± 3 cells/mm^2, $P < 0.05$). The proportion of CD3-positive cells producing TNFα was similar across the 3 groups (32% (76/234), 33% (30/90), 35% (3.5/10)) (**Figure 4.6 A**). There was no difference between the 3 groups with regard to the percentage area of the sections stained with intravascular TNFα (**Figure 4.6 B**). Stromal cell TNFα staining was present in 10/10 patients with active ocular MMP and 7/10 patients with treated ocular MMP (**Table 4.2**). Occasional stromal cells stained positive in 3/10 normal controls. Epithelial TNFα staining was present in 4/10 patients with active ocular MMP, 4/10 patients with treated ocular MMP, and 1/10 normal controls.

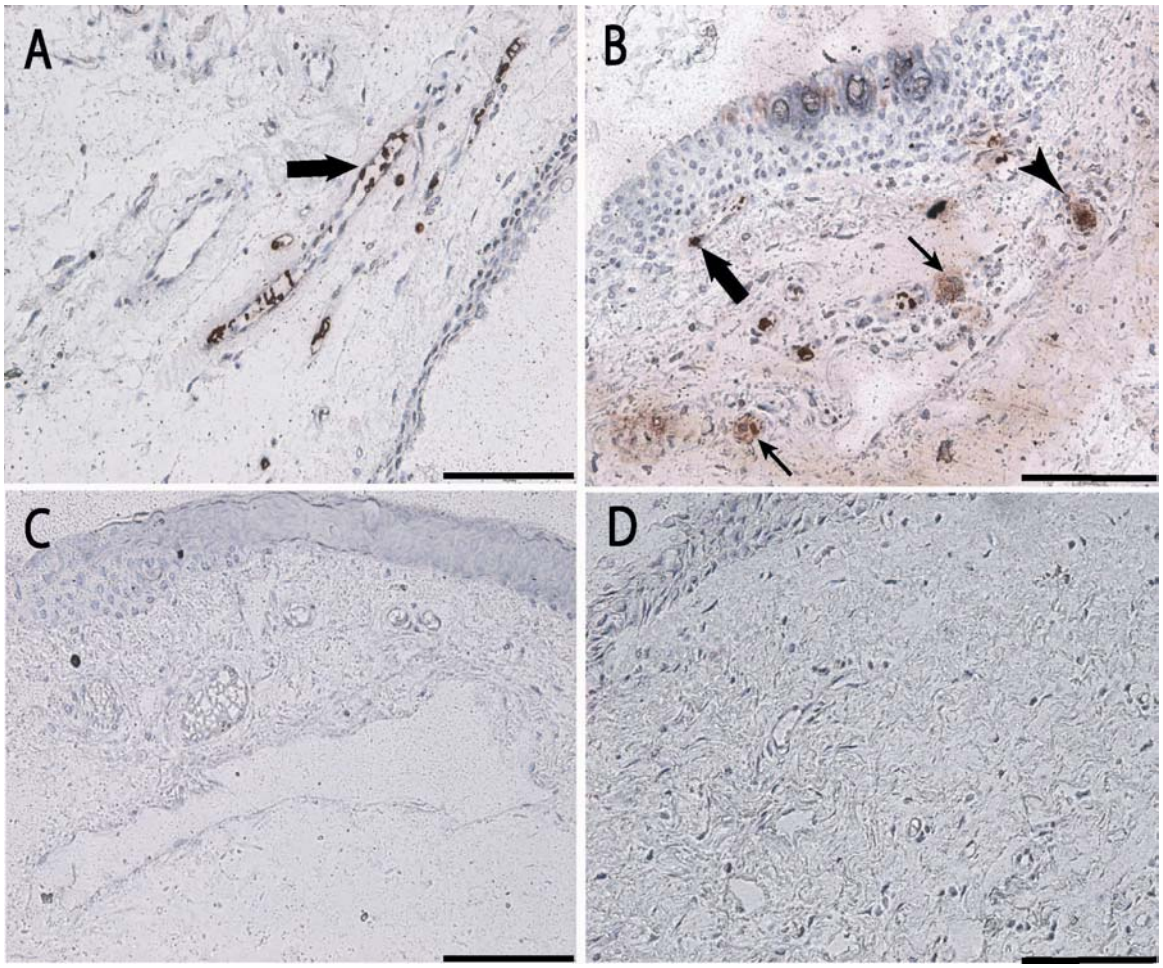


Figure 4.5 Human conjunctiva of mucous membrane pemphigoid patients shows tumour necrosis factor-alpha (TNFα) expression.
Immunohistochemistry of bulbar conjunctival sections showing **A.** Intravascular TNFα+CD3- cells in a normal subject, **B.** Positive intravascular and stromal TNFα staining in a patient with actively inflamed ocular MMP. Small arrows indicate stromal TNFα+CD3- cells, arrowhead indicates stromal TNFα+CD3+ cell, large arrow indicates intravascular TNFα+CD3- cell. TNFα stained dark red (AEC), CD3 stained brown (DAB). **C.** a negative isotype mAb control of a normal subject, **D.** a negative isotype mAb control of a patient with actively inflamed ocular MMP. Note the stromal inflammatory cell infiltrate. Bar = 100μm

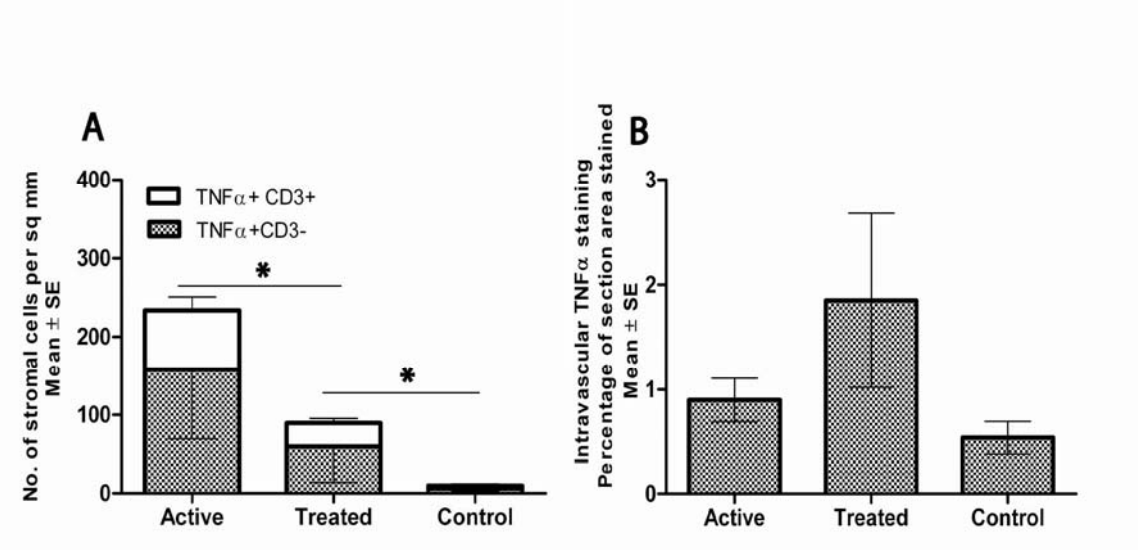


Figure 4.6 Cell counts of positive stromal staining in human conjunctiva of ocular MMP patients and controls. A. Stromal TNFα/ CD3 double-staining cell counts. Many stromal TNFα-expressing cells are present in patients with active ocular MMP (234 cells/mm^2), 32% (76/234) of which also express the T cell marker CD3. Following immunosuppressive therapy, in clinically uninflamed treated ocular MMP, the number of stromal TNFα-expressing cells is significantly reduced to 38% of the number of cells present in active disease (90 cells/mm^2, *P* <0.05), but this is still 9-fold greater than the number of stromal TNFα-expressing cells present in normal controls (10 cells/mm^2, P <0.05). **B.** Percentage area of the sections stained with intravascular TNFα. There is no difference in intravascular TNFα staining between active, treated ocular MMP, or controls. Results are the mean and SE from 10 individuals per group, counting at least 9 fields per individual. * *P* <0.05.

Table 4.2 Stromal and epithelial TNFα staining results for each patient

Diagnosis	Case number	Stromal TNFα staining (grade N to ++++)	Epithelial TNFα staining (grade N to ++++)
Active MMP	1	+++	N
	2	+	+++
	3	+++	++
	4	+++	+++
	5	++	N
	6	++	N
	7	++++	N
	8	++++	++++
	9	++	N
	10	+	N
Treated uninflamed MMP	1	++	+
	2	+ / N	N
	3	+ / N	N
	4	+	+
	5	N	N
	6	++	++
	7	N	N
	8	+	N
	9	N	N
	10	++	+
Normal control	1	N	N
	2	N	N
	3	N	N
	4	+ / N	N
	5	N	N
	6	+ / N	N
	7	N	N
	8	+ / N	+
	9	N	N
	10	N	N

MMP= mucous membrane pemphigoid, N = nil staining

4.4.2 Normal conjunctival fibroblast migration, collagen contraction and proliferation in response to TNFα

All the experiments described from this point onwards in this chapter were carried out on normal human conjunctival fibroblasts.

The effect of TNFα on key fibroblast functions including cell migration, collagen contraction and proliferation was investigated. Whilst TNFα stimulated conjunctival fibroblast migration under serum-free conditions ($P < 0.0001$, **Figure 4.7**), no significant contraction of fibroblast-collagen lattices was observed in response to a range of TNFα concentrations [1, 10, 100ng/ml] compared with the serum-free negative control (data not shown). There was also no significant difference in fibroblast cell division after stimulation with 10ng/ml TNFα, compared with the FCM negative control (data not shown).

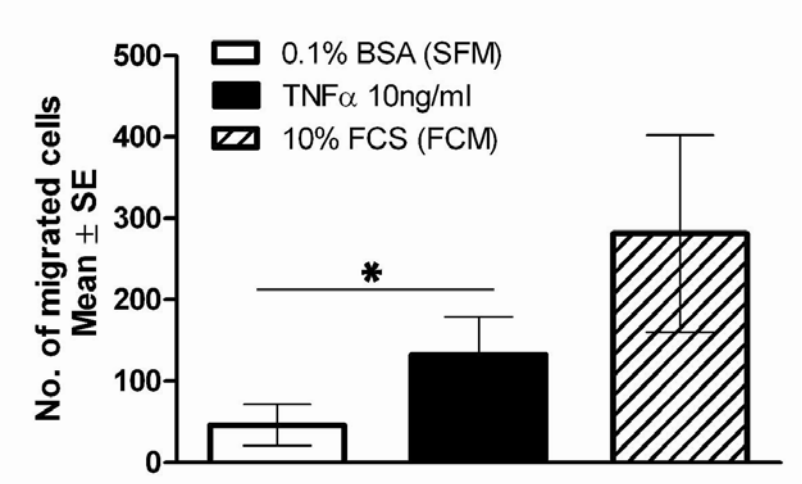


Figure 4.7. Effect of TNFα on conjunctival fibroblast migration. Conjunctival fibroblasts were seeded into culture inserts incorporating porous membranes and allowed to migrate overnight towards the lower chamber containing the test substance. The negative control used was serum-free medium (SFM) containing 0.1% bovine serum albumin (BSA), the positive control used was fibroblast culture medium (FCM) containing 10% foetal calf serum. TNFα stimulated fibroblast migration under serum-free conditions. * P <0.0001.

4.4.3 Matrix metalloproteinase protein production in response to TNFα

Conditioned media from fibroblast-populated collagen lattices treated with TNFα were analysed using an antibody-coated membrane array testing a panel of 7 mmps and 3 timps. **Figure 4.8** shows that the level of mmp-9 increased (*P* =0.01) and the levels of timp-2 (*P* =0.01) and timp-4 decreased (*P* =0.04) in the presence of TNFα, compared with the serum-free negative control.

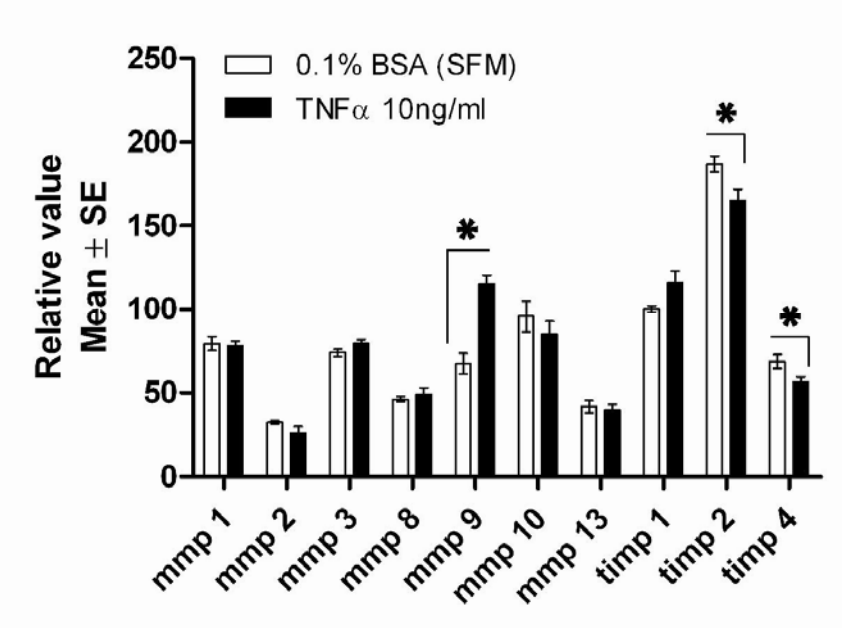


Figure 4.8. Matrix metalloproteinase (mmp) and tissue inhibitor of matrix metalloproteinase (timp) levels in conditioned medium of conjunctival fibroblast-populated collagen gels incubated over 7 days in the presence of TNFα, as detected by antibody-coated membrane array. Data are relative values compared with the membrane array negative control. There was a significant increase in mmp-9 (*P* =0.01) and a decrease in timp-2 (*P* = 0.01) and timp-4 (*P* = 0.04) levels in the conditioned medium from TNFα-treated collagen gels, in comparison with the untreated control 0.1% bovine serum albumin (BSA) serum-free medium (SFM) collagen gels. Results are the mean and SE from 5 individual donors. * *P* < 0.05

4.4.4 Immune Marker Expression by Conjunctival Fibroblasts in response to TNFα

Interactions between tissue fibroblasts and infiltrating T lymphocytes via the CD40/CD40L pathway have been shown to powerfully costimulate T lymphocyte proliferation and to activate fibroblasts, thus potentially promoting fibrogenesis (Sempowski *et al.*, 1997). Surface expression of HLA-DR, ICAM and T cell-costimulatory molecules by conjunctival fibroblasts in response to 72hours' stimulation with TNFα or IFNγ under serum-free conditions was investigated.

ICAM was constitutively expressed by a high percentage of fibroblasts (84.3% ± 13.5%) under basal conditions, and there was no significant change in the percentage level of expression after TNFα treatment (**Figure 4.9 A**). However, the intensity of ICAM expression by the fibroblasts escalated in response to increasing concentrations of TNFα (mean fluorescence intensity in the negative control BSA (bovine serum albumin) 113 ± 29, 1ng/ml TNFα 1361 ± 402, 10ng/ml TNFα 2117 ± 604, 100ng/ml TNFα 2133 ± 717, $P = 0.03$ linear regression, **Figure 4.9 B**). Fibroblast expression of the costimulatory molecule CD40 was significantly upregulated by TNFα, with the maximum percentage expression occurring in response to 10ng/ml TNFα (BSA 1.8% ± 1.3%, 1ng/ml TNFα 14.3% ± 8.3%, 10ng/ml TNFα 27.7% ± 11.7%, $P = 0.04$, Fig. 6A). No significant change in either the percentage or intensity of expression of HLA-DR, CD80, CD86 or CD40-ligand was detected. In comparison, IFNγ upregulated expression of HLA-DR, CD40 and CD154.

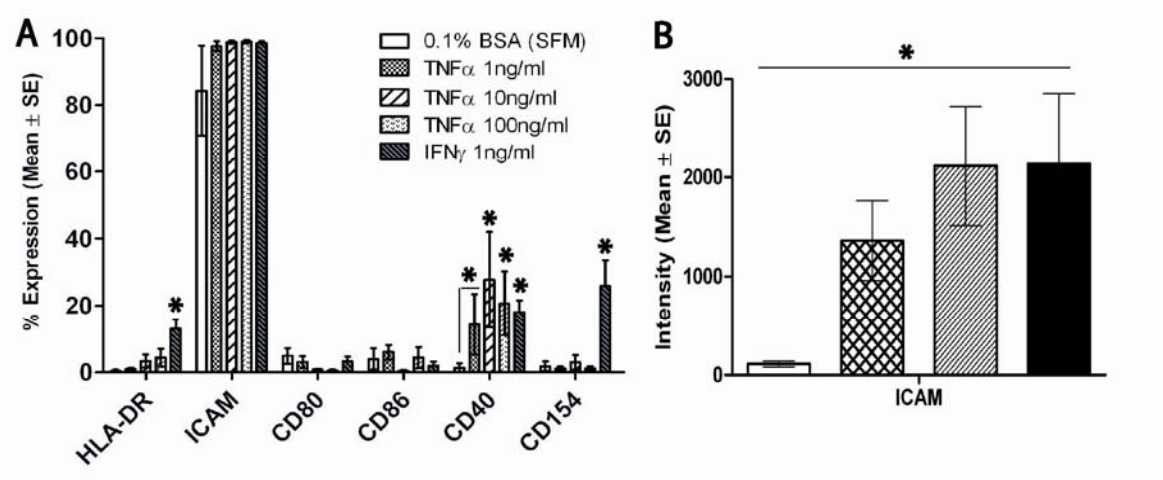


Figure 4.9 Fibroblast expression of surface markers of activation and costimulatory molecules in response to TNFα. Conjunctival fibroblasts were treated with various concentrations of TNFα [1, 10, 100 ng/ml] or IFNγ [1ng/ml] for 72 hours under serum-free conditions, and the surface expression of HLA-DR, ICAM, CD80, CD86, CD40, and CD40-ligand was determined by flow cytometry. The negative control was serum-free medium (SFM) containing 0.1% bovine serum albumin (BSA). **A.** Percentage of conjunctival fibroblasts expressing surface markers and costimulatory molecules. All concentrations of TNFα significantly upregulated the percentage expression of CD40 (*P <0.05 in comparison to untreated control), with maximum expression occurring in response to 10ng/ml. Note the high percentage of fibroblasts expressing ICAM under basal conditions and in the presence of TNFα. **B.** Intensity of expression of ICAM (intercellular adhesion molecule) by conjunctival fibroblasts in response to TNFα stimulation. Whilst the percentage of cells expressing ICAM was high under basal conditions and in the presence of TNFα, the intensity of ICAM expression increased in response to TNFα stimulation (* P =0.03). Results are the mean ± SE from 6 individual donors.

4.5 Discussion

The results in this chapter demonstrate increased tissue expression of TNFα in ocular MMP, thus systemic treatment with TNFα antagonists could be expected to ameliorate inflammatory activity in this disease. Furthermore, it has been demonstrated that whilst systemic immunosuppressive treatment is associated with a significant decrease in tissue TNFα expression, there is residual persistent expression of TNFα even when inflammation clinically appears to be controlled. A possible role for TNFα in the pathogenesis of conjunctival fibrosis in mucous membrane pemphigoid has also been demonstrated, in that TNFα is a chemoattractant for conjunctival fibroblasts. Migration of fibroblasts to the site of injury expressing TNFα could lead to a pathological accumulation of fibroblasts and excessive scarring at this site. TNFα increases mmp-9 production, and causes activation of conjunctival fibroblasts by upregulating the intensity of adhesion molecule expression and upregulating CD40 expression.

No difference in cell division in response to 10ng/ml TNFα in FCM compared with FCM alone was detected in the experiments presented here, but proliferation of myofibroblasts in response to TNFα has previously been reported (Porter *et al.*, 2004; Theiss *et al.*, 2005). The normal conjunctival fibroblasts used in the experiments in this chapter had previously been assessed for myofibroblast characteristics using alpha-smooth muscle actin staining looking for assembled actin myofilaments, and these had been found to be absent (data not shown). Different TNFα concentrations may have stimulated proliferation in our assay, or it may be that TNFα- stimulated proliferation is a myofibroblast-specific response.

TNFα has previously been reported to be present in 6/8 ocular MMP patients in a limited study by Coma et al; it is not clear whether these patients had active inflammation, or clinically uninflamed disease (Cordero *et al.*, 2007). Bernauer et al previously found positive substantia propria expression of TNFα in all active MMP,

chronic MMP and normal conjunctival specimens (Bernauer *et al.*, 1993a); this may have been due to intravascular staining which was similarly found, in the results reported in this chapter, to be present in all specimens. In the results reported in this chapter, intravascular staining was evaluated separately from extravascular stromal TNFα staining, and the latter was found to be virtually absent in normal conjunctiva.

The findings in this chapter of persistent residual TNFα expression, even when the conjunctiva is clinically 'white' and uninflamed after systemic immunosuppressive treatment, is in agreement with previous work proposing ongoing release of cytokines in the presence of a significant cellular infiltrate ('white inflammation') which cannot be seen on the slit-lamp (Elder, 1997c). Progressive fibrosis despite immunosuppressive treatment may be driven by this underlying chronic inflammation.

The results in this chapter support a profibrotic and proinflammatory effect of TNFα on conjunctival fibroblasts. The free-floating fibroblast-populated collagen lattice model is thought to represent contraction resulting from fibroblast migration through matrix (Grinnell, 2000). Although addition of TNFα did not stimulate matrix contraction, increased mmp-9 levels were detected in the conditioned medium of the fibroblast-populated collagen lattices. These findings are consistent with other studies reporting TNFα-induced mmp-9 expression by gingival fibroblasts and other cells (Kim *et al.*, 2008; Porter *et al.*, 2004). Leonardi et al have shown increased mmp-9 and also mmp-1 production, and decreased timp-1 production in response to TNFα stimulation, by conjunctival fibroblasts adherent to a tissue culture plate (Leonardi *et al.*, 2003). They did not examine timp-2 or -4 expression. The differences in mmp-1 and timp-1results between my study and theirs may reflect differences in the in vitro models used. The in vitro model of fibroblasts populating a 3-dimensional collagen gel matrix used could be seen as more physiological than fibroblasts cultured in a monolayer on plastic.

Matrix-metalloproteinase-9 (also known as gelatinase-B) degrades extracellular matrix collagens such as collagen type IV, the major component of basement membranes, and laminin, plays a role in regulating cell migration and cleaves latent TGFβ. The mmp-9 detected by the antibody array in this study could have been either the latent or active form. Recently it has been shown that the proteolytic activities of mmps are not necessary for mmp-induced cell migration, and that the hemopexin domain of pro-mmp-9 plays an important role in cell migration (Dufour *et al.*, 2008). The results of the migration assay experiments in this chapter, showing increased fibroblast migration in response to TNFα, may well be related to this increased mmp-9 production, noting that this would represent migration without concomitant matrix contraction, given that no contraction of the collagen lattices was observed in response to TNFα. Decreased timp-2 and timp-4 levels were also detected in the conditioned medium of the fibroblast-populated lattices. By virtue of their ability to inhibit mmp activity, all timps are believed to function as inhibitors of angiogenesis. Timp-2 has also been found to directly inhibit endothelial cell proliferation and angiogenesis by an mmp-independent effect (Stetler-Stevenson & Seo, 2005). Timp-4 has a growth promoting activity in vitro, prevents apoptosis, and inhibits TNFα converting enzyme (Wong *et al.*, 2002). It is possible that the ability of TNFα to stimulate angiogenesis is related to this reduction in timp activity.

T lymphocytes are significantly increased in the subepithelial cellular infiltrate across all phases of ocular MMP disease activity (Bernauer *et al.*, 1993b), and T cell-fibroblast cross talk and feedback loops may be an important mechanism in fibrosis. IFNγ-stimulated lung fibroblasts have been shown to costimulate T lymphocyte proliferation utilizing CD40, but not via CD80 or CD86 costimulatory molecules (Sempowski *et al.*, 1997). Interactions between tissue fibroblasts and infiltrating T lymphocytes, via the CD40/CD40 ligand pathway, augment inflammation and may promote fibrogenesis by activating both cell types.The

results in this chapter show that TNFα upregulates expression of CD40 on conjunctival fibroblasts, which may then interact with T cells via CD40 ligand, inducing the fibroblasts to proliferate, produce cytokines, and lay down extracellular matrix (Kaufman *et al.*, 2001), as well as stimulating T cell proliferation and production of cytokines which could promote further fibroblast activation and matrix deposition. The effect of the addition of TNFα on functional fibroblast activity in the presence of T cell co-culture requires further investigation. The majority of the TNFα-expressing cells were not T cells, and are likely to be monocytes/macrophages. This should be confirmed by further experiments.

4.6 Conclusion

In summary, expression of TNFα is increased in the conjunctiva of patients with ocular mucous membrane pemphigoid, hence systemic TNFα antagonists could be expected to be useful in severe ocular mucous membrane pemphigoid not responding to conventional immunosuppressants. TNFα appears to have a profibrotic effect on normal conjunctival fibroblasts. However, these profibrotic effects appear to have a limited scope, given that TNFα does not appear to cause collagen matrix contraction, which is a key aspect of wound contraction and remodelling. Furthermore, TNFα is likely to be mostly produced by macrophages and neutrophils, while most of the subacute and chronic inflammatory cell infiltrate in ocular MMP is composed of T cells. Th2- polarized responses are recognized as playing an important role in fibrogenesis. In searching for key molecules or mechanisms involved in fibrosis in ocular MMP, assessing the role of T cells and potentially important T cell-derived cytokines is thus pertinent.

Chapter 5

Interleukin-13 (IL-13) expression in ocular mucous membrane pemphigoid, its effects on conjunctival fibroblasts and T cell studies in ocular mucous membrane pemphigoid

5.1 Introduction

The findings in Chapter 4 indicate that TNFα plays a role in tissue inflammation in ocular MMP, and has some profibrotic effects on conjunctival fibroblasts, but these effects are modest. The aim of this thesis was to investigate potential key molecules and mechanisms involved in conjunctival fibrosis in ocular MMP, so given its prominent role in driving fibrosis in chronic inflammatory liver and lung disease, the Th2-type cytokine IL-13 was a prime candidate for investigation.

Fibrosis associated with chronic inflammation is unique in that the adaptive immune response, and in particular a type 2 helper T cell (Th2)-polarized response, is thought to play an important role (Wynn, 2008). As in most fibroproliferative disorders (Azouz *et al.*, 2004), both type 1 (Th1) (transforming growth factor-β, IFNγ) and type 2 (Th2) cytokines (IL-4, IL-5) are present in mucous membrane pemphigoid lesions (Bernauer *et al.*, 1993a; Caproni *et al.*, 2003; Elder *et al.*, 1997; Razzaque *et al.*, 2003a), but it is not known whether the Th2 cytokine IL-13 is present. Given the predominance of T cells in the substantia propria infiltrate in ocular MMP, investigating their potential role in ocular MMP fibrosis by characterising their cytokine secretion profile including IL-13 secretion, and evaluating the effect of ocular MMP T cell-fibroblast co-culture on conjunctival fibroblast and T cell activity was of interest. T cell-fibroblast interactions may promote a chronic inflammation-fibrosis cycle in ocular MMP.

Inhibition of the effects of IL-13 using a recombinant soluble form of IL-13Rα2 has been reported (Andrews *et al.*, 2006). This soluble receptor, which lacks transmembrane and cytoplasmic domains, has inhibited IL-13-mediated responses in a dose-dependent manner. The mechanism by which this occurs is believed to be by binding IL-13 with high affinity and acting as a non-signalling decoy receptor.

Preliminary steps in assessing the potential importance of IL-13 and T cells in fibrosis in ocular MMP therefore include establishing whether IL-13 is expressed in ocular MMP, investigating the direct effects of IL-13 on conjunctival fibroblasts (given the potentially different functional characteristics of fibroblasts derived from different anatomical sites (Flavell *et al.*, 2008), attempting to inhibit the effects of IL-13 using an IL-13 inhibitor, and characterising the T cells in ocular MMP.

5.2 Aim

The aim of the study in this chapter was to investigate whether IL-13 is expressed in conjunctival MMP tissue including after systemic immunosuppressive treatment, to assess the effects of IL-13 on normal human conjunctival fibroblasts, and to characterise the T cells in ocular MMP conjunctiva and evaluate potential T cell-fibroblast interactions.

5.3 Research design and methods

5.3.1 Conjunctival biopsies

Bulbar conjunctival biopsies were obtained from ocular MMP patients and patients with normal conjunctiva as described in **Chapter 4, section 4.3.1**. Details of the patients (11 active ocular MMP, 10 treated uninflamed ocular MMP) and 9 normal controls whose conjunctival biopsies were used in the IL-13 experiments in this chapter are shown in **Table 5.1**.

Table 5.1 Details of patients and normal controls whose conjunctival biopsies were used in IL-13 studies

Diagnosis	Case number	Age	Gender	Disease duration (yrs)	Eye biopsied	Bulbar inflammation grade (0 to 4)	Tauber stage (Upper stage/ Lower stage)	Topical therapy	Systemic therapy	Other eye pathology
	1	54	F	0.5	L	3	IIcIIId/ IIcIIId	hypromellose, acetylcysteine, yellow soft paraffin	nil	
	2	59	M	3	L	2.5	I/ IIa	hypromellose, lacrilube	nil	
	3	76	M	5	L	3	IIaIIIa(1)/IIbIIIb(2)	hypromellose, chloramphenicol	mycophenolate	amblyopia
	4	80	M	10	R	3	IIbIIIc(2)/ IIdIIId(2)	carmellose	mycophenolate + dapsone	
	5	55	M	13	3	IIcIIIc(2)/ IIdIIId(1)	dexamethasone, carmellose	mycophenolate + dapsone + deflazocort		
Active MMP	6	60	F	2	R	3	I/ IIcIIIc(3)	carmellose	mycophenolate + dapsone	
	7	76	F	2	R	2.5	IIc/ IIbIIIa(2)	prednisolone, hypromellose, carmellose	mycophenolate + dapsone	sicca, blepharitis
	8	51	F	0.1	L	3	I/ IIIa(1)	nil	nil	
	9	57	M	0.5	L	3	IIb/ IIcIIIb(2)	dexamethasone, lacrilube	nil	
	10	64	F	1	R	2	IIa/ IIbIIIc(2)	prednisolone	nil	
	11	83	F	7	L	3	IIa/ IIbIIIb(2)	brimonidine, latanoprost, timolol, dorzolamide, carmellose	mycophenolate + doxycycline	glaucoma
	1	59	F	10	R	1	IIbIIIb(2)/ IIdIIId(2)	carbomer 980	dapsone	
	2	86	F	15	R	1	IIb/ IIcIIIb(2)	nil	nil	
	3	78	F	10	R	1	IIbIIIb(2)/ IIdIIId(2)	nil	nil	
	4	84	F	2	L	0	IIb/ IIcIIIc(2)	nil	mycophenolate	
Treated uninflamed MMP	5	66	F	2	R	0	IIb/ IIcIIIb(2)	nil	dapsone	
	6	59	M	4	L	1	I/ IIa	hypromellose, carmellose, liquid paraffin, chloramphenicol	cyclophosphamide	
	7	76	M	6	L	1.5	IIdIIId(2)/ IIbIIIb(2)	chloramphenicol, hypromellose, retinoic acid, acetylcysteine	cyclophosphamide	blepharitis
	8	60	F	3	L	1	I/IIaIIIa(1)	carmellose	cyclophosphamide + dapsone	
	9	62	F	0.75	R	0.5	IIa/ IIcIIIa(1)	hyaluronate	prednisolone	
	10	51	F	1	L	1	I/ IIIa(1)	nil	cyclophosphamide + prednisolone	
	1	50	F	-	L	0	-	nil	nil	cataract
	2	76	M	-	L	0	-	nil	nil	cataract
	3	65	M	-	R	0	-	nil	nil	cataract
	4	70	F	-	R	0	-	nil	nil	cataract
Normal control	5	82	F	-	L	0	-	nil	nil	cataract
	6	65	M	-	R	0	-	nil	nil	cataract
	7	75	F	-		0	-	nil	nil	cataract
	8	73	F	-		0	-	nil	nil	cataract
	9	62	M	-	R	0	-	nil	nil	cataract

5.3.2 Immunohistochemistry

Immunohistochemistry was carried out on GMA-embedded sections in an identical manner to that described in **Chapter 4, section 4.3.2**, apart from substituting IL-13 for TNFα and using different primary and secondary antibodies. The primary IL-13 antibody was a rabbit antibody against human IL-13 (Biogenesis Ltd, Poole UK [5378-8530]), diluted 1:100. The secondary antibody used for this was a biotinylated goat anti-rabbit immunoglobulin (Dako, Cambridgeshire UK), diluted 1:200. For IL13-CD3 double staining, IL-13 was stained red (AEC) and CD3 stained brown (DAB), Human tonsil sections were used as positive controls, and the two negative controls used were absence of the primary antibody and an isotype matched, irrelevant monoclonal antibody (Dako). The number of cells stained in the stroma were counted in a masked fashion in 5 or more high power fields per patient using a Leica DM RBE microscope at x400 magnification with a 10mm^2 eyepiece graticule (Leica, Milton Keynes UK). Counts were expressed as mean counts per mm^2. The presence of positive stromal or epithelial staining was also graded on a scale of 0 to ++++, based on both the number of cells stained and staining intensity.

5.3.3 Isolation of Normal Conjunctival Fibroblasts

Normal conjunctival fibroblasts were grown from the conjunctival biopsies and passaged as described in **Chapter 4, section 4.3.3.**

To investigate the effect of IL-13 on direct profibrotic activity by normal human conjunctival fibroblasts, the effect of IL-13 on collagen contraction, matrix metalloproteinase and type I collagen secretion, fibroblast proliferation and migration was assessed. A concentration of 10ng/ml IL-13 was used initially for all assays, based on previously published data (Fujitsu *et al.*, 2005; Leonardi *et al.*, 2003) showing a response at this concentration. If no response was observed, a

dose-titration experiment was set up to determine the optimum IL-13 concentration for that particular assay.

5.3.4 Collagen contraction model

Free-floating relaxed fibroblast-populated collagen gels were prepared as described in **Chapter 4, section 4.3.5.** The lattices were detached immediately after feeding with 10ng/ml IL-13 (recombinant human IL-13, R&D systems, Abingdon, UK) in SFM. SFM alone was used as the negative control, and 10% gelatinase-free serum-containing FCM was used as the positive control. The gels were digitally photographed at days 1, 3 and 7, and the gel areas were calculated using image analysis software (Image J).

IL-13 Inhibition

Blockade of the effects of IL-13 on collagen contraction was attempted, using a recombinant soluble form of IL-13Rα2 acting as a decoy receptor (monoclonal anti-IL-13Rα2 from Diaclone, IDS, Boldon, Tyne and Wear, UK), as described by Andrews et al (Andrews *et al.*, 2006). These authors had also shown that 24 hours of pre-treatment with IL-13 induced high surface expression of IL-13Rα2 on fibroblasts, and this then attenuated the cell responses to IL-13. For this reason, soluble IL-13Rα2 [1µg/ml] was added 24 hours after commencement of the collagen contraction assays, which had been fed with IL-13 [10ng/ml] at commencement. The combined addition of IL-13 [10ng/ml] with IL-13Rα2 [1µg/ml or 10µg/ml] at commencement of the collagen contraction assays was also tested. The assays of IL-13 inhibition were conducted over 3 days rather than 7 days, because it was believed unlikely that the inhibitor would be continuing to have an effect beyond 3 days.

5.3.5 Matrix metalloproteinase protein levels

Conditioned medium collected from the collagen lattice experiments between days 0-3 and 3-7, or days 0-7, was analysed for matrix metalloproteinase-1 (mmp-1), mmp-2, mmp-3, mmp-8, mmp-10, mmp-13, tissue inhibitor of matrix

metalloproteinase-1 (timp-1), timp-2 and timp-4 protein levels as described in **Chapter 4, section 4.3.6**.

5.3.6 Type I collagen secretion

Conditioned medium collected from contracting lattices under both serum-free and serum-containing conditions, in the presence and absence of IL-13 [10ng/ml], was analysed for secretion of the C-terminal propeptide of type I collagen (CICP) using an ELISA (Quidel Corp, San Diego CA) carried out according to the manufacturer's instructions.

5.3.7 Proliferation

Fibroblast cell division in the presence or absence of IL-13 was analysed by flow cytometry using the fluorescent probe CFSE (carboxyfluorescein diacetate, succinimidyl ester) as described in **Chapter 4, section 4.3.7**. The CFSE-containing cells were seeded into 6-well tissue culture plates at a density of 4 x 10^5 cells per well and stimulated with IL-13 10ng/ml in FCM.

5.3.8 Migration

Fibroblast migration experiments were conducted as described in **Chapter 4, section 4.3.4**, in the presence and absence of IL-13 [10ng/ml], with or without the addition of T cell culture supernatant (see below). SFM was used as a negative control, and 10% bovine serum FCM as a positive control.

<u>T cell culture supernatant</u>

Supernatant from a T cell line culture, derived from a conjunctival biopsy from an atopic keratoconjunctivitis patient, and stimulated with phytohaemagglutinin, was were obtained from previous experiments. Previous ELISA testing had indicated that this supernatant contained IL-5 and IFNγ. This supernatant was tested as a chemoattractant in the lower chamber of the tissue culture plate at a concentration of 1:10 in SFM, either alone or in combination with IL-13 [10ng/ml].

5.3.9 Immunofluorescence staining and flow cytometry

Fibroblasts may actively engage in perpetuating chronic inflammation via direct fibroblast-T cell cross talk, and IL-13 may play a role in stimulating this activity. Surface expression of markers of activation (HLA-DR, ICAM-1) and costimulatory molecules (CD80, CD86, CD40, CD40 ligand) by normal conjunctival fibroblasts in response to stimulation by a range of concentrations of IL-13 [1, 10, 100ng/ml] and IFNγ [1ng/ml] (recombinant human IFNγ, Peprotech, London UK) for 72 hours was evaluated by flow cytometry in an identical manner to that described in **Chapter 4, section 4.3.8**.

5.3.10 T cell studies

Experiments to grow T cells from conjunctival biopsies and peripheral blood, based on a previously described method (Calder *et al.*, 1999) were attempted.

T cell culture from conjunctival biopsies

Fresh conjunctival biopsies were placed into 1ml T cell medium with 1µg/ml PHA (phytohaemagglutinin;Sigma-Aldrich) and 20% human IL-2 (Lymphocult-T, Biotest Folex, Solihull, UK). T cell medium was composed of RPMI 1640 (Dutch modification), 2mM L-glutamine, 2mM NEAAs non-essential amino acids, 2mM sodium pyruvate, 50µg/ml gentamicin, 2 x 10^{-5}M 2-Mercaptoethanol and 10% heat inactivated human AB+ serum (all from Gibco, Paisley, UK). 5% to 20% IL-2 was added every 3 days and the cells were restimulated with 1µg/ml PHA and mitomycin-C treated JY feeder cells weekly (2:1 ratio of feeders: T cells). Mitomycin-C treated JY feeder cells were prepared by incubating 50µg/ml mitomycin-C in RPMI to the required number of JY cells for 90 minutes at 37^{o}C then washing the cells 3 times in RPMI by centrifuging at 1200 rpm for 10 minutes. The aim was to maintain cells at approximately 2 x 10^{5} cells/ml, in a 2ml T cell medium volume per well, of a 24 well plate. At least 4 rounds of feeder cell/PHA stimulation were performed before carrying out experiments to characterise the T

cells phenotypically, measure cytokine production or intracellular cytokine production.

Peripheral blood mononuclear cell (PBMC) culture

6-12ml heparinised peripheral blood was diluted 1:1 with RPMI 1640, then layered over Histopaque 1077 (Sigma-Aldrich) and centrifuged at 2400rpm for 20 minutes. Following centrifugation, the mononuclear cell layer was extracted from between the yellow plasma and clear Histopaque layers with a sterile pipette. PBMCs were washed twice with RPMI by centrifuging at 1200rpm for 10 minutes. The cells were either used immediately for culture or cryopreserved.

If used immediately for culture, the number of cells was counted and then plated at approximately 2 x 10^5 cells/ml into wells of a 24-well plate containing 2ml T cell medium in each well, and 1µg/ml PHA was added. 5% to 20% IL-2 was added every 3 days and the cells were restimulated with 1µg/ml PHA and mitomycin-C treated JY feeder cells weekly (2:1 ratio of feeders: T cells).

Cells were cryopreserved at a concentration of approximately 2 x 10^6/ml in 1ml T cell medium or RPMI containing 10% foetal calf serum, placed on ice and dimethylsulfoxide (DMSO) was added dropwise to a final concentration of 10%. The cryovials were immediately stored in an insulated box at -70^{o}C then 3-4 days later transferred into liquid nitrogen.

5.3.11 Statistical Analysis

Differences between 2 groups were examined for statistical significance using the Mann-Whitney U test and one-way analysis of variance (ANOVA) was used to compare the means of 3 or more unmatched groups. $P < 0.05$ was considered to be statistically significant.

5.4 Results

5.4.1 IL-13 is expressed in mucous membrane pemphigoid conjunctiva, in both active disease and clinically uninflamed treated disease

There was minimal expression of IL-13 in the substantia propria of normal conjunctiva (**Figure 5.1 B**). In contrast, in active conjunctivitis due to mucous membrane pemphigoid there was a significant increase in the number of stromal cells expressing IL-13 (39 ± 1 cells/mm^2) (**Figure 5.1 D**), 80% (31/39) of which were also positive for the T cell marker CD3 (**Figure 5.2**). Following immunosuppressive therapy, in clinically uninflamed treated mucous membrane pemphigoid patients, the number of stromal IL-13-expressing cells was significantly reduced to 44% (17/39) of the number of cells present in active disease (17 ± 0.5 cells/mm^2, $P < 0.001$), but this was still 4-fold greater than the number of stromal IL-13-expressing cells present in normal conjunctiva (4 ± 0.3 cells/mm^2, $P < 0.001$). The proportion of CD3-positive cells was similar across the 3 groups (80% (31/39), 76% (13/17), 75% (3/4) (**Figure 5.2).** Stromal cell IL-13 staining was present in 11/11 patients with active ocular MMP and 10/10 patients with treated ocular MMP (**Table 5.2**). Occasional stromal cells stained positive in 5/9 normal controls. Epithelial IL-13 staining was present in 7/11 patients with active mucous membrane pemphigoid, 9/10 patients with treated mucous membrane pemphigoid, and 5/9 normal controls.

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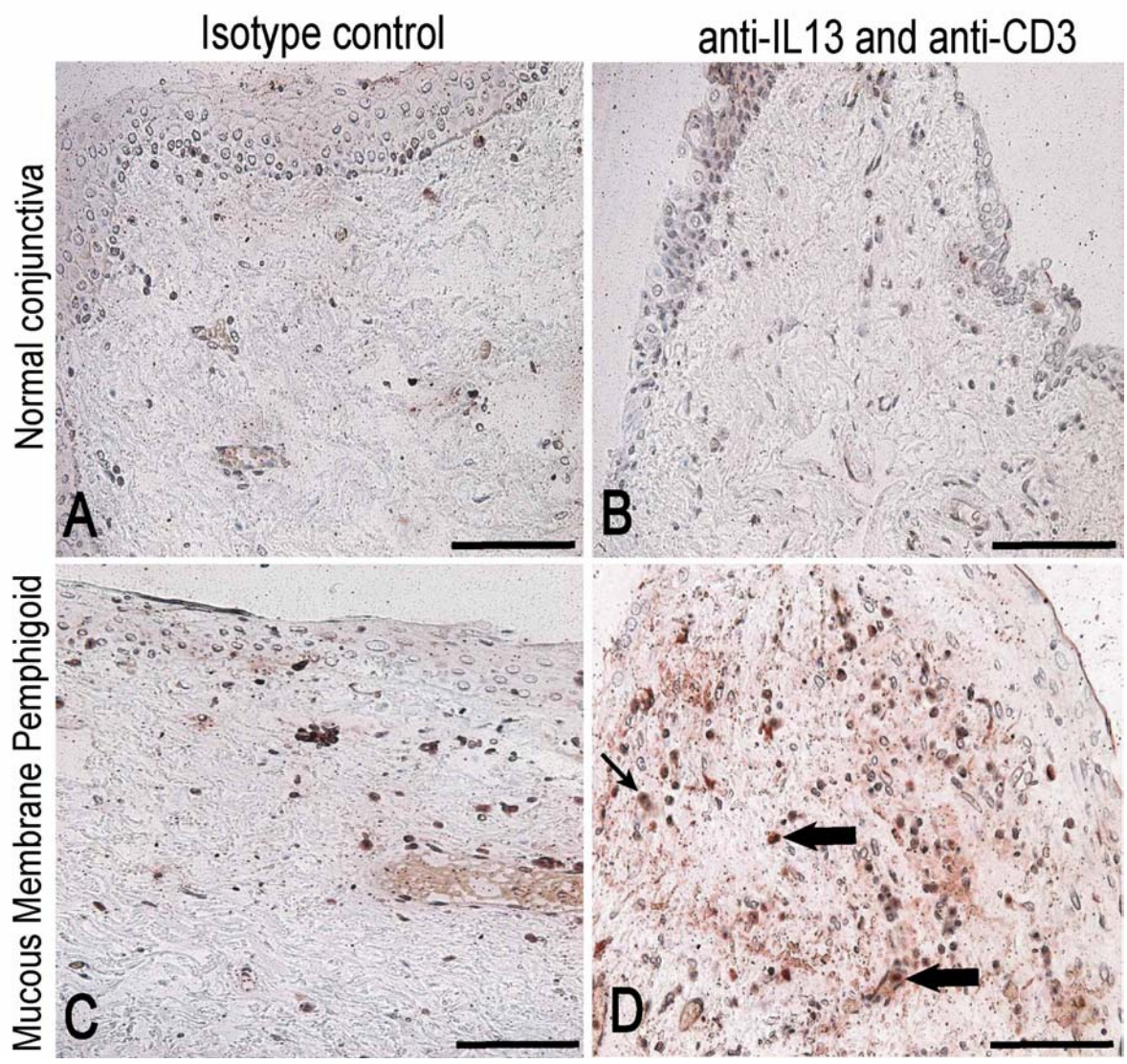

Figure 5.1 Human conjunctiva of ocular MMP patients shows IL-13 expression.
Immunohistochemistry of bulbar conjunctival sections showing **A.** a negative isotype mAb control of a normal subject, **B.** very little IL-13 or CD3 staining in a normal subject, **C.** a negative isotype mAb control of a patient with actively inflamed mucous membrane pemphigoid, **D.** positive IL-13 (red, AEC) and CD3 (brown, DAB) double staining in a patient with actively inflamed ocular MMP. Thick arrows indicate double-stained IL-13+CD3+ cells, thin arrow indicates single-stained IL13-CD3+ cell. Bar = 100μm. AEC amino-ethyl carbazole, DAB 3,3'diaminobenzidine.

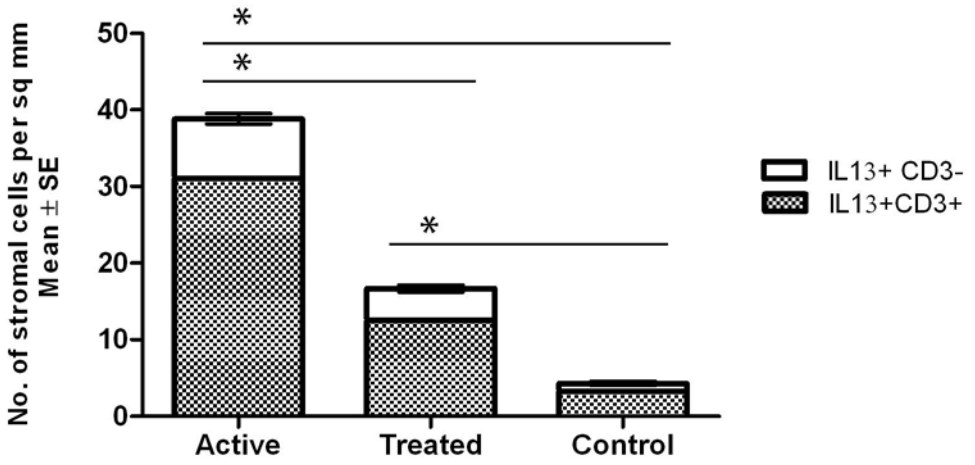

Figure 5.2 Cell counts of positive stromal IL-13/ CD3 staining in MMP conjunctiva and normal controls. Many stromal IL-13-expressing cells are present in patients with active ocular MMP (39 ± 1 cells/mm^2), 80% (31/39) of which also express the T cell marker CD3. Following immunosuppressive therapy, in clinically uninflamed treated ocular MMP, the number of stromal IL-13-expressing cells is significantly reduced to 44% (17/39) of the number of cells present in active disease (17 ± 0.5 cells/mm^2), but this is still 4-fold greater than the number of stromal IL-13-expressing cells present in normal controls (4 ± 0.3 cells/mm^2). *P < 0.001. Results are the mean and SE from at least 9 individuals per group, counting at least 9 fields per individual.

Table 5.2 Stromal and epithelial IL-13 staining results for each patient

Diagnosis	Case number	Stromal IL-13 staining (grade N to ++++)	Epithelial IL-13 staining (grade N to ++++)
Active MMP	1	+++	++++
	2	++++	++
	3	++	+/ N
	4	++++	++
	5	+++	+
	6	+++	N
	7	+	N
	8	++	N
	9	+++	N
	10	++++	+++
	11	++	+/ N
Treated uninflamed MMP	1	++	+++
	2	+	+
	3	+	N
	4	+	+
	5	+	+++
	6	+	+++
	7	+	++
	8	+	++
	9	++	+++
	10	++	+++
Normal control	1	++	++
	2	N	+
	3	N	++
	4	+/ N	N
	5	+/ N	N
	6	+/ N	N
	7	N	+
	8	N	N
	9	+/ N	+

MMP= mucous membrane pemphigoid, N = nil staining

5.4.2 IL-13 stimulates collagen contraction by normal human conjunctival fibroblasts, which is associated with decreased mmp-3 and mmp-10 secretion

IL-13 was found to stimulate collagen contraction by normal human conjunctival fibroblasts (**Figure 5.3**). Attempts to inhibit this collagen contraction using soluble IL-13Rα2 showed promising results on the preliminary dose-titration results (**Figure 5.4 A**), but when the experiments were repeated at the selected concentration of 1μg/ml, there was no significant difference between the results with or without the inhibitor, either following pre-treatment with IL-13 for 24 hours (**Figure 5.4 B**), or with simultaneous addition of IL-13Rα2 at the commencement of the assay (data not shown). Using an increased IL-13Rα2 concentration of 10μg/ml also did not show any significant difference (**Figure 5.4 C**).

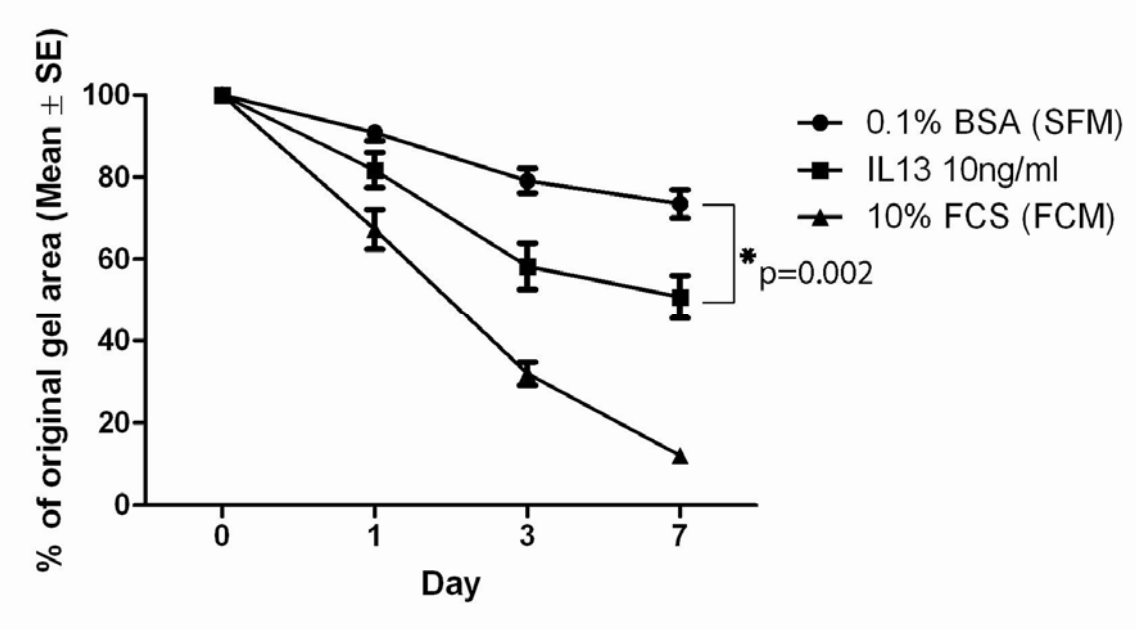

Figure 5.3 Contraction of fibroblast-populated collagen lattice gels by normal human conjunctival fibroblasts in response to the addition of IL-13 under serum-free conditions. The untreated negative control was 0.1% bovine serum albumin (BSA) serum-free medium (SFM). Fibroblast culture medium (FCM) containing 10% foetal calf serum (FCS) was used as a positive control. Results are the mean and SE from 6 or more individual donors. IL-13 stimulates collagen contraction by normal human conjunctival fibroblasts.

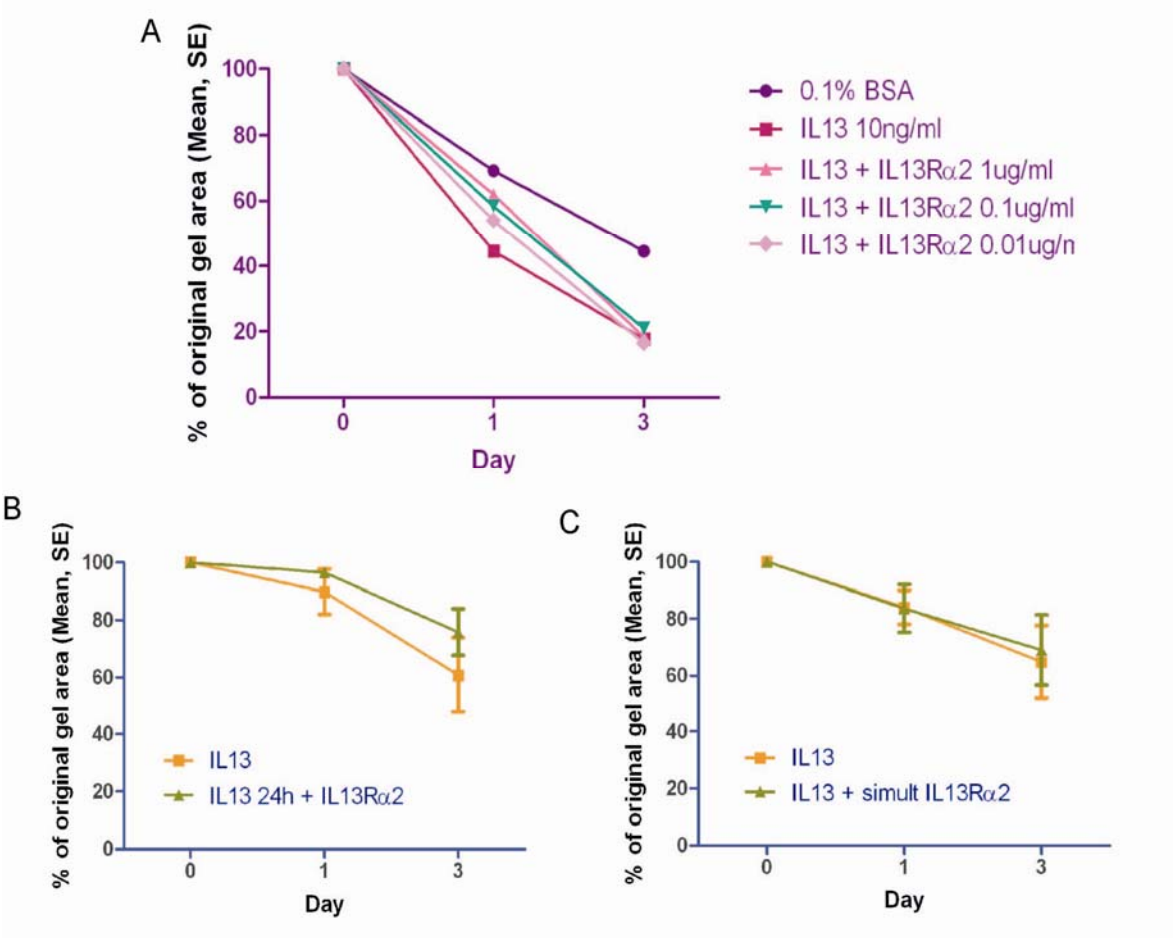

Figure 5.4 Attempted neutralization of IL-13 by soluble IL-13Rα2. A. Preliminary dose-response curve showing inhibition of IL-13-mediated collagen contraction when IL-13Rα2 was added 24hours after commencement of the assay. The IL-13Rα2 concentration of 1µg/ml was selected for further experiments based on these results. **B.** Further experiments showed no difference in gel contraction with or without 1µg/ml of the soluble inhibitor. Results are the mean and SE of 4 individual donors. **C.** Addition of IL-13Rα2 at the commencement of the assay (rather than 24hours later) also showed no difference, either at a concentration of 1µg/ml (data not shown) or 10µg/ml (data shown). Results are the mean and SE of 5 individual donors.

Secretion of mmp-3 and mmp-10 during collagen contraction was decreased in the presence of IL-13 (**Figure 5.5**).

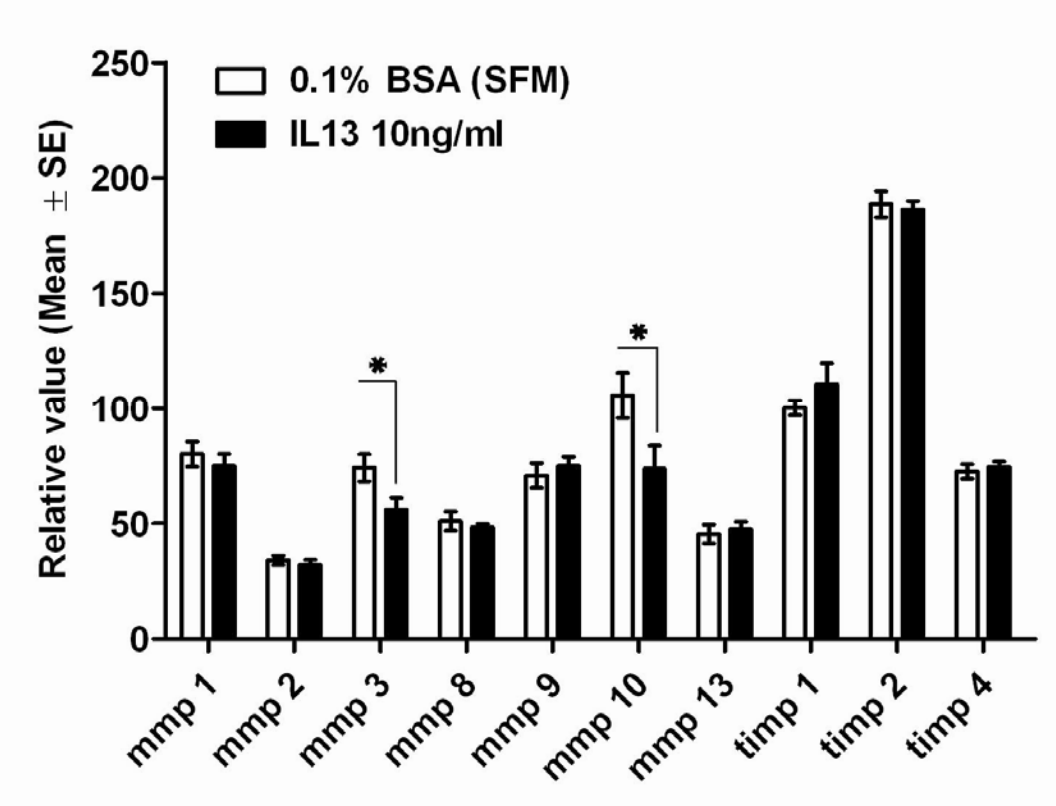

Figure 5.5 Matrix metalloproteinase (mmp) and tissue inhibitor of matrix metalloproteinase (timp) levels in conditioned medium of conjunctival fibroblast-populated collagen gels incubated over 7 days in the presence of IL-13, as detected by antibody-coated membrane array. Data are relative values compared with the membrane array negative control. There was a significant decrease in mmp-3 (P =0.04) and mmp-10 (P = 0.01) levels in the conditioned medium from IL-13-treated collagen gels, in comparison with the untreated control of 0.1% bovine serum albumin (BSA) serum-free medium (SFM). Results are the mean and SE from 6 individual donors. * $P < 0.05$

5.4.3 IL-13 stimulates chemotaxis of normal conjunctival fibroblasts. No detected change in collagen secretion or proliferation

IL-13 was also found to stimulate chemotaxis of normal human conjunctival fibroblasts (**Figure 5.6**).

No significant change in type I collagen production was detected in response to the addition of 10ng/ml IL-13 in both serum-free and serum-containing collagen lattice experiments (data not shown). No significant differences in fibroblast proliferation were observed in the presence of 10ng/ml IL-13 compared with the FCM negative control (data not shown).

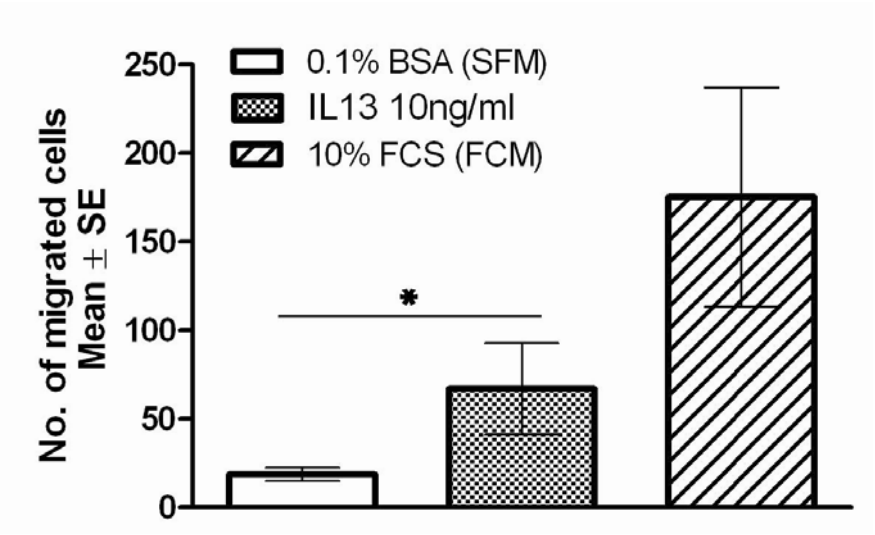

Figure 5.6 Effect of IL-13 on normal human conjunctival fibroblast migration. Conjunctival fibroblasts were seeded in the upper chambers of porous membranes and allowed to migrate overnight towards the lower chamber containing the test substance. The negative control used was 0.1% bovine serum albumin (BSA) serum-free medium (SFM), the positive control used was 10% foetal calf serum-containing fibroblast culture medium (FCM). IL-13 stimulated fibroblast migration under serum-free conditions. Results are the mean and SE of 5 individual experiments. * P = 0.01.

5.4.4 IL-13 augments the stimulatory effect of T cell culture supernatant on fibroblast chemotaxis

To evaluate whether IL-13 had synergistic effects, in combination with T cell-derived mediators, on normal human conjunctival fibroblasts, the effect of addition of T cell culture supernatant to the chemotaxis assay was investigated. The combination of IL-13 and T cell culture supernatant significantly augmented the effect of either treatments alone (**Figure 5.7**).

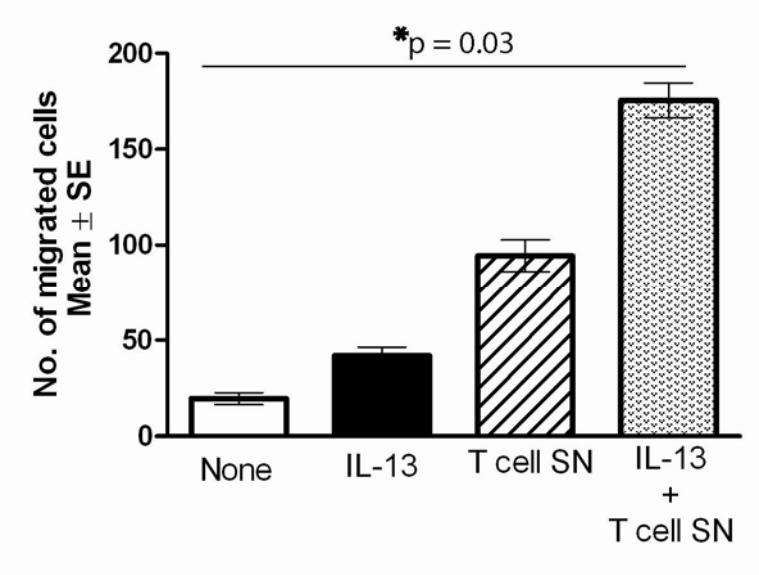

Figure 5.7 Effect of IL-13 combined with T cell supernatant on normal human conjunctival fibroblast migration. The negative control used was 0.1% bovine serum albumin (BSA) serum-free medium (SFM). T cell supernatant (SN) was used at 1:10 concentration, and IL-13 at 10ng/ml. The combination of IL-13 and T cell supernatant acted synergistically to significantly enhance fibroblast migration. Results are the mean and SE of 3 individual experiments. * *P* = 0.03, one way ANOVA.

5.4.5 IL-13 upregulates surface expression of HLA-DR, CD80, CD40 and CD154 on normal human conjunctival fibroblasts

ICAM-1 was constitutively expressed by a high percentage of fibroblasts (84.3% ± 13.5%), and there was no significant change in the percentage of cells expressing ICAM in the presence of IL-13 (**Figure 5.8**). In contrast, HLA-DR, CD80, CD40 and CD154 were minimally expressed under basal serum-free conditions (HLA-DR 0.7% ± 0.4%, CD80 7.2% ± 2.5%, CD40 0.3% ± 1.8%, CD154 1.28% ± 2.0%), and all were significantly upregulated in response to stimulation by IL-13, but only at the maximum concentration of 100ng/ml (HLA-DR 7.5% ± 3.9% P = 0.01, CD80 12.7% ± 9.4% P = 0.04, CD40 52.8% ± 9.9% P = 0.03, CD154 22.4% ± 5.6% P = 0.03, **Figure 5.8 and Figure 5.9**). No significant change in either the percentage expression of CD86 was detected, nor in the intensity of expression of ICAM-1 (data not shown). As a comparison, interferon-gamma (IFNγ) 1ng/ml was found to significantly upregulate HLA-DR, CD40 and CD154 expression, but not CD80 (**Figure 5.8**).

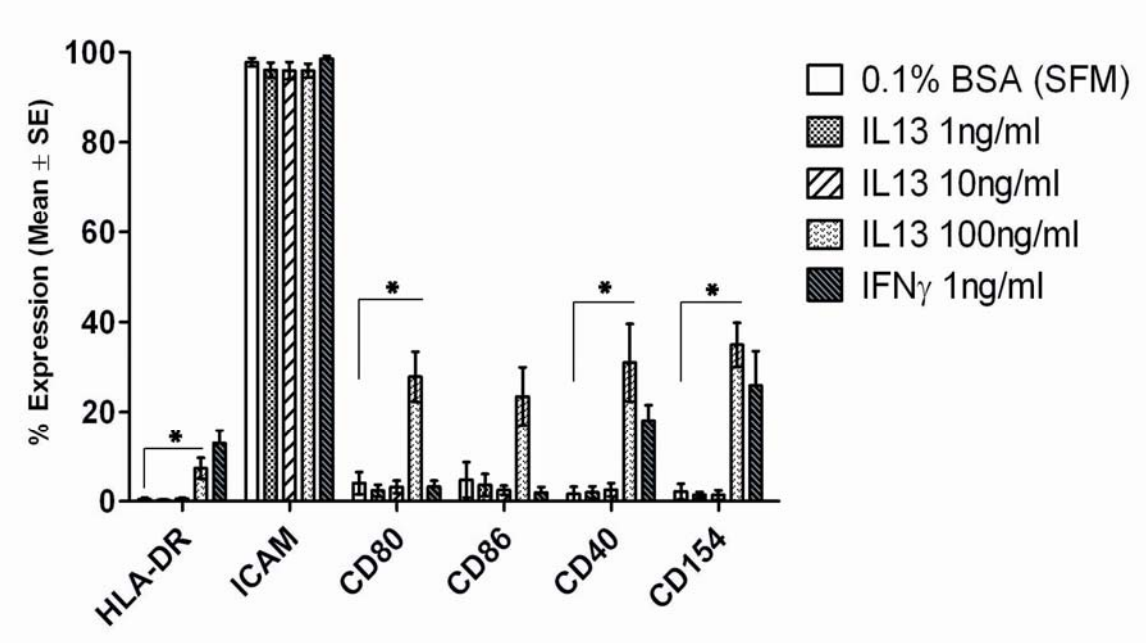

Figure 5.8 Fibroblast expression of surface markers of activation and costimulatory molecules in response to IL-13. Normal human conjunctival fibroblasts were treated with various concentrations of IL-13 [1, 10, 100 ng/ml] or IFNγ [1ng/ml] for 72 hours under serum-free conditions, and the percentage of conjunctival fibroblasts expressing on their cell surface HLA-DR, ICAM, CD80, CD86, CD40, and CD154 was determined by flow cytometry. Serum-free medium (SFM) containing 0.1% bovine serum albumin (BSA) was the negative control. 100ng/ml IL-13 significantly upregulated the surface expression of HLA-DR, CD80, CD40 and CD154. As a comparison, 1ng/ml IFNγ upregulated HLA-DR, CD40 and CD154 expression. Results are the mean ± SE from 6 individual donors. *$P < 0.05$.

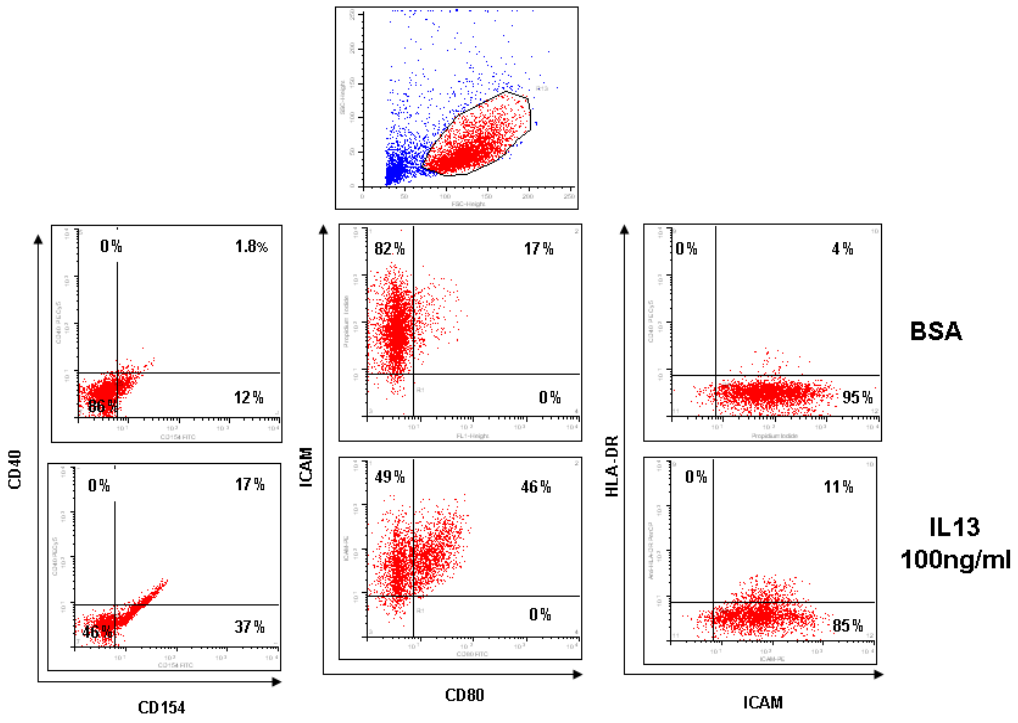

Figure 5.9 Representative density plots of surface marker expression flow cytometry. Upregulated surface expression of CD40, CD154, CD80 and HLA-DR was observed in the presence of 100ng/ml IL-13, compared with the untreated control 0.1% bovine serum albumin (BSA) serum-free medium (SFM), by a normal human conjunctival fibroblast primary cell line.

5.4.6 T cell studies

Attempts to culture T cells from the conjunctival biopsies were unsuccessful, in part due to fungal contamination and in part due to inability to maintain cell survival. Attempts to grow peripheral blood mononuclear cells, for the purpose of T cell-fibroblast co-culture experiments, were similarly unsuccessful.

5.5 Discussion

The results in this chapter show that IL-13 is expressed in the conjunctiva in the scarring condition mucous membrane pemphigoid, and that it has direct effects in stimulating normal human conjunctival fibroblast collagen contraction and migration, and modifying matrix metalloproteinase secretion *in vitro*, which could lead to connective tissue remodelling. IL-13 may also promote the active involvement of these cells in pro-inflammatory activities via upregulating HLA-DR, CD80, CD40, and CD154 expression, thus enabling the fibroblasts to cross-talk with lymphocytes and other inflammatory cells. IL-13 appears to act synergistically with lymphocyte-secreted products to augment chemotaxis of conjunctival fibroblasts.

The findings in this chapter of increased collagen contraction and migration in response to IL-13 are similar to reports from other studies where human lung fibroblasts were investigated (Kohyama *et al.*, 2004; Liu *et al.*, 2002). Reduced matrix metalloproteinase- 3 secretion by normal human conjunctival fibroblasts in response to IL-13 has similarly been described by Fukuda et al (Fukuda *et al.*, 2006), but they did not investigate matrix metalloproteinase-10 secretion, which we also found was reduced by IL-13. Moreover, they also found increased release of timp-2, which was not detected by our assay method. These differences are probably due to different culture conditions. Matrix metalloproteinase-3 (mmp-3) is a stromelysin which cleaves pro-mmps 1,7,8,9,and 13, laminin and fibronectin (Wong *et al.*, 2002). It is thus a key regulator of mmp activity, and reduced secretion of mmp-3 is consistent with reduced degradation of extracellular matrix and a net increase in collagen. Matrix metalloproteinase-10 is also a stromelysin, and has similar activities to mmp-3.

The unsuccessful inhibition of IL-13 induced collagen contraction by soluble IL-13Rα2 may have been because of several reasons: (1) the soluble IL-13Rα2 needed to be pre-incubated with IL-13 alone, prior to adding it to the fibroblasts, in order for the soluble inhibitor to achieve a sufficient reduction of IL-13. Andrews et al carried out this preincubation for 6 hours. This was not done for the experiments in this study because it had been felt that, in the event of the soluble IL-13Rα2 being utilized as therapy, the preincubation was unlikely to be representative of a physiological interaction in the tissues; (2) the concentration of soluble IL-13Rα2 was not high enough to bind and prevent IL-13 from attaching to its active receptor on the fibroblasts; (3) the 24 hour pre-incubation with IL-13 was not successful in attenuating the response to IL-13 because the cells had not been washed after the 24hour incubation and then re-stimulated with IL-13. Finding suitable IL-13 inhibitors that work both *in vitro* and *in vivo* requires further investigation.

An exciting preliminary observation from this study is that IL-13 upregulates expression of the T cell costimulatory molecules CD80, CD40 as well as CD154 (CD40 ligand) on human conjunctival fibroblasts, and also upregulates HLA-DR expression. In comparison, IFNγ upregulated HLA-DR, CD40 and CD154 expression. This upregulated surface molecule expression in response to IL-13 and IFNγ could have been confirmedby using additional methods such as Western blotting, immunocytochemistry and/ or laser confocal microscopy of fibroblasts seeded in glass chamber slides, real time PCR for mRNA expression, and immunohistochemistry of pemphigoid conjunctival tissues investigating the expression of these molecules.

Whilst the CD40/ CD154 pathway has been implicated in the development of fibrosis in the lung, it has not previously been investigated with respect to conjunctival fibroblasts. The CD40/ CD154 interaction is a potent activator of fibroblasts, and we hypothesise that the promotion of autocrine and paracrine activation of conjunctival fibroblasts via this pathway by IL-13, which upregulates

both expression of the ligand (CD40) and the receptor (CD154) on these cells, plays a role in the perpetuation of inflammation and wound healing responses in mucous membrane pemphigoid. Upregulated expression of CD154 in human lung fibroblasts by IL-13 has previously been reported by Kaufman et al (Kaufman *et al.*, 2004), who detected CD154 more readily intracellularly than on the surface of lung fibroblasts. It is possible that they were less able to detect surface CD154 because they used trypsin to detach fibroblasts for flow cytometry rather than collagenase. Trypsin cleaves surface molecules more readily than collagenase (Bluml, 2007). In contrast to Kaufman's findings however, the results presented here showed that IFNγ also upregulated CD154 expression by fibroblasts. The findings in this chapter regarding persistently elevated expression of IL-13 in conjunctiva that appeared 'white' and clinically uninflamed following systemic immunosuppressive treatment but that still showed progressive fibrosis, are consistent with fibroblasts playing an active role in perpetuating this chronic inflammation.

Upregulated expression of CD80 (B7-1) on conjunctival fibroblasts was also found in the results presented here. Whilst binding of CD80 to its receptor CD28 on T cells is an essential costimulatory step in T cell activation, there is an inhibitory receptor on T cells for the B7 ligands, CTLA-4 (cytotoxic T lymphocyte- associated antigen 4 / CD152) (Bhatia *et al.*, 2006), which is upregulated after T cell activation has peaked, and limits the degree of activation. It is possible that engagement of the inhibitory receptor CTLA-4 on T cells, by conjunctival fibroblasts expressing CD80, regulates the activation process. Tesavibul et al have previously reported significantly high CD86 (B7-2) expression in the conjunctival substantia propria of mucous membrane pemphigoid compared with normal conjunctiva, and minimal detection of CD80 (Tesavibul *et al.*, 1998). The CD86 positive cells were thought to be mostly tissue macrophages and Langerhans' cells, and the differences in CD80/ CD86 expression may also reflect differences in regulation of expression, given that B7-1 (CD80) is expressed in a tightly regulated manner but B7-2 (CD86) is constitutively expressed on the cell surface of antigen presenting cells.

The principal mediators responsible for the observed synergistic enhancement of the chemotactic effect of IL-13 with T cell supernatant are most likely IL-2, IL-4, IL-5 and/ or IFNγ (Crowston *et al.*, 1997). Antibody neutralization experiments would aid in identifying the responsible mediators. It is possible that other actions of IL-13 on fibroblasts also require synergism with other growth factors. The unsuccessful T cell culture experiments meant that unfortunately further understanding of the relative importance of IL-13 and T cells in conjunctival fibrosis was limited.

In contrast with a previous study (Fujitsu *et al.*, 2005), an increase in proliferation of normal conjunctival fibroblasts in response to IL-13 was not able to be detected in the experiments presented here. This may have been due to differences in the method used. In addition, use of different IL-13 concentrations may have stimulated proliferation in our assay. Leonardi et al have detected proliferation of conjunctival fibroblasts derived from vernal keratoconjunctivitis (VKC) patients in response to IL-13 using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Leonardi *et al.*, 2003), but difficulty in getting the MTT assay to work led to the use of CFSE flow cytometry in our experiments. Leonardi also showed increased procollagen I production in response to IL-13 by VKC fibroblasts grown on culture plates in fresh ascorbic acid with 0.4% serum. Unlike the findings of Leonardi et al, no change in collagen secretion in the conditioned medium from contracting collagen lattices was detected in the results presented here, either under serum-free conditions or in the presence of 10% heat inactivated serum. There could be several reasons for the difference in findings: in our experiments the fibroblasts were actively contracting the collagen lattices and thus unlikely to divert their metabolic activities to synthesizing collagen at the same time, or alternatively the medium conditions were not optimal for collagen synthesis, or because of phenotypic differences between VKC and normal conjunctival fibroblasts, or because IL-13 alone has only a modest effect on

collagen synthesis, and synergism with another factor such as TGF-β is necessary (Zhou *et al.*, 2005).

Developing effective therapies to inhibit the profibrotic effects of IL-13 on fibroblasts and other inflammatory cells will involve understanding the relative roles and actions of the IL-13 receptors and their signalling pathways. There are 2 different IL-13 receptor chains to which IL-13 binds (Hershey, 2003). First, the IL13Rα1 chain, which combines with the IL-4 receptor α-chain, to form a dimeric transmembrane IL-13 receptor which is expressed on IL-13-responsive cells, and is activated not only by IL-13, but also by IL-4. Secondly, the IL-13Rα2 chain, which binds IL-13 with high affinity, but was formerly considered to only function as a decoy receptor. Recently it has been shown in macrophages that IL-13 signaling via induction of cell-surface expression of IL-13Rα2, activates the TGF-β1 promoter to synthesise TGF-β and cause fibrosis (Fichtner-Feigl *et al.*, 2006). Induction of IL-13Rα2 requires the presence of both IL-13 (or IL-4) and TNFα. The results in chapter 4 showed that TNFα is expressed in ocular mucous membrane pemphigoid, and it is possible that the presence of both cytokines (TNFα and IL-13) activates TGF-β production and conjunctival fibrosis via this mechanism. A synergistic effect of TNFα and IL-13 on expression of VCAM-1 (vascular cell adhesion molecule-1) by corneal fibroblasts has also been reported (Kumagai *et al.*, 2003).

5.6 Conclusion

IL-13 is expressed in actively inflamed and uninflamed conjunctiva in ocular MMP. It is likely to contribute to conjunctival fibrosis in mucous membrane pemphigoid via both indirect mechanisms involving TGFβ and other cytokines, and via direct effects on conjunctival fibroblast contractility, migration and mmp secretion. By upregulating the ability for cross-talk between conjunctival fibroblasts and other inflammatory cells, IL-13 may facilitate fibroblast-generated chronic inflammation and continued fibrosis.

Chapter 6

Conjunctival fibroblasts from patients with mucous membrane pemphigoid appear to have a profibrotic phenotype

6.1 Introduction

Chapters 4 and 5 highlighted the roles of the inflammatory cytokines TNFα and IL-13 in acute and chronic inflammation in ocular MMP. Whilst inflammation typically precedes the development of fibrosis in most fibrotic disorders, some experimental models suggest that fibrosis is not always characterized by persistent inflammation, and that to a degree, the mechanisms regulating fibrosis are distinct from those controlling inflammation (Stramer *et al.*, 2007). In line with this viewpoint, an alternative theory for the observed progression of fibrosis in ocular MMP is that the fibroblasts have been transformed into an abnormally activated phenotype, or this abnormal phenotype has been preferentially selected and expanded, in a similar fashion to the profibrotic phenotype observed and maintained *in vitro* in fibroblasts from patients with systemic sclerosis, pulmonary, renal and colonic fibrosis (Ramos *et al.*, 2001; Schuttert *et al.*, 2003; Trojanowska, 2004).

There is some evidence of phenotypic changes in fibroblasts isolated from mucous membrane pemphigoid patients (Razzaque *et al.*, 2003b; Razzaque *et al.*, 2004; Roat *et al.*, 1989), but whether there are pathological alterations in key aspects of fibroblast behaviour such as motility, contractile function, matrix synthesis, and development of myofibroblast characteristics, has not been investigated. Moreover, whether there are differences in fibroblasts isolated from actively inflamed tissue compared with fibroblasts isolated from clinically uninflamed tissue, is unknown.

6.2 Aim

The study in this chapter sought to establish whether there are phenotypic differences in fibroblast functional activity between mucous membrane pemphigoid conjunctival fibroblasts and normal conjunctival fibroblasts, and whether fibroblasts isolated from actively inflamed tissue differ from those isolated from clinically uninflamed tissue.

6.3 Research Design and Methods

6.3.1 Conjunctival biopsies

Bulbar conjunctival biopsies were obtained from 11 active ocular MMP patients, 8 uninflamed ocular MMP patients, and from 11 patients with normal conjunctiva as described in **Chapter 4, section 4.3.1**. Details of the patients and controls from whom biopsies were used for the experiments in this chapter are shown in **Table 6.1**.

6.3.2 Isolation of Normal Conjunctival Fibroblasts

Normal conjunctival fibroblasts, actively inflamed pemphigoid conjunctival fibroblasts and uninflamed pemphigoid conjunctival fibroblasts were grown from the conjunctival biopsies and passaged as described in **Chapter 4, section 4.3.3.**

6.3.3 Proliferation

Fibroblast cell division was analysed by flow cytometry using the fluorescent probe CFSE (carboxyfluorescein diacetate, succinimidyl ester) as described in **Chapter 4, section 4.3.7**. The CFSE-containing cells were seeded into 6-well tissue culture plates at a density of 4 x 10^5 cells per well in 10% serum-containing FCM and cultured for 96 hours.

6.3.4 Migration

Fibroblast migration experiments were conducted as described in **Chapter 4, section 4.3.4**, in either (1) SFM, or (2) 10% serum-containing FCM.

Table 6.1 Details of patients and normal controls whose conjunctival biopsies were used in pemphigoid fibroblast studies

Diagnosis	Age	Gender	Disease duration (yrs)	Bulbar inflammation grade (0 to 4)	Tauber stage (Upper stage/ Lower stage)	Topical therapy	Systemic therapy	Other eye pathology
Active MMP	54	F	0.5	3	IIcIIId/ IIcIIId	hypromellose, acetylcysteine, yellow soft paraffin	nil	
	80	M	10	3	IIbIIIc(2)/ IIdIIId(2)	carmellose	mycophenolate + dapsone	
	55	M	13	3	IIcIIIc(2)/ IIdIIId(1)	dexamethasone, carmellose	mycophenolate + dapsone + deflazocort	
	74	M	2	2.5	IIa/ IIcIIIc(2)	dorzolamide, bimatoprost	nil	glaucoma
	76	F	2	2.5	IIc/ IIbIIIa(2)	prednisolone, hypromellose, carmellose	mycophenolate + dapsone	sicca, blepharitis
	81	M	2	2.5	IIaIIIa(1)/ IIbIIIb(2)	carmellose	mycophenolate + dapsone	
	83	M	1	3	IIbIIIa(1)/ IIcIIIc(2)	ofloxacin, chloramphenicol	nil	
	62	F	18	2	IIcIIIc(2)/IIdIIId(2)	timolol, carbomer 980	mycophenolate	glaucoma
	64	F	1	2	IIa/ IIbIIIc(2)	prednisolone	nil	
	83	F	7	3	IIa/ IIbIIIb(2)	brimonidine, latanoprost, timolol, dorzolamide, carmellose	mycophenolate + doxycycline	glaucoma
	51	F	0.1	3	I/ IIIa(1)	nil	nil	
Treated uninflamed MMP	59	F	10	1	IIbIIIb(2)/ IIdIIId(2)	carbomer 980	dapsone	
	86	F	15	1	IIb/ IIcIIIb(2)	nil	nil	
	78	F	10	1	IIbIIIb(2)/ IIdIIId(2)	nil	nil	
	84	F	2	0	IIb/ IIcIIIc(2)	nil	mycophenolate	
	66	F	2	0	IIb/ IIcIIIb(2)	nil	dapsone	
	66	F	2	0	IIb/ IIcIIIb(2)	nil	dapsone	
	76	M	6	1.5	IIdIIId(2)/ IIbIIIb(2)	chloramphenicol, hypromellose, retinoic acid, acetylcysteine	cyclophosphamide	
	60	F	3	1	I/IIaIIIa(1)	carmellose	cyclophosphamide + dapsone	
Normal control	50	F	-	0	-	nil	nil	cataract
	76	M	-	0	-	nil	nil	cataract
	65	M	-	0	-	nil	nil	cataract
	70	F	-	0	-	nil	nil	cataract
	65	M	-	0	-	nil	nil	cataract
	75	F	-	0	-	nil	nil	cataract
	73	F	-	0	-	nil	nil	cataract
	57	M	-	0	-	nil	nil	cataract
	65	M	-	0	-	nil	nil	cataract
	79	F	-	0	-	nil	nil	cataract
	62	M	-	0	-	nil	nil	cataract

MMP mucous membrane pemphigoid, F = female, M = male

6.3.5 Collagen contraction model

Free-floating relaxed fibroblast-populated collagen gels were prepared as described in **Chapter 4, section 4.3.5.** The lattices were detached immediately after feeding with either (1) SFM, (2) 10ng/ml IL-13 in SFM (recombinant human IL-13, R&D systems, Abingdon, UK), (3) 10ng/ml TNFα in SFM (recombinant human TNFα, R&D systems, Abingdon, UK), or (4) fibroblast culture medium (FCM) containing 10% gelatinase-free calf serum (Gibco). The gels were digitally photographed at days 1, 3 and 7, and gel areas were calculated using image analysis software (Image J).

6.3.6 Matrix metalloproteinase protein levels

Conditioned medium collected from the collagen lattice experiments between days 0-3 and 3-7, and days 0-7, was analysed for matrix metalloproteinase-1 (mmp-1), mmp-2, mmp-3, mmp-8, mmp-10, mmp-13, tissue inhibitor of matrix metalloproteinase-1 (timp-1), timp-2 and timp-4 protein levels as described in **Chapter 4, section 4.3.6**.

6.3.7 Type I collagen secretion

Conditioned medium collected from contracting lattices was analysed for secretion of the C-terminal propeptide of type I collagen (CICP) using an ELISA (Quidel Corp, San Diego CA) carried out according to the manufacturer's instructions.

6.3.8 Chemokine secretion

To investigate whether pemphigoid fibroblasts are more secretory than normal fibroblasts, and have the potential to draw in large numbers of inflammatory cells and thus cause a greater degree of conjunctival inflammation by secreting chemoattractant molecules called chemokines, we evaluated secretion of chemokines following 72 hours' stimulation with key ocular MMP cytokines. Confluent fibroblasts in 6 well culture plates were cultured in SFM for 24h then

stimulated for 72 hours with a panel of cytokines in 10% heat-inactivated serum-containing FCM.

The cytokines selected were those reported to be present in mucous membrane pemphigoid, including transforming growth factor-β1 (TGFβ1; 50ng/ml), IL-4 20ng/ml, interferon-γ (IFNγ; 200ng/ml), TNFα 10ng/ml, and IL-13 100ng/ml. The culture supernatants were analysed for secretion of the chemokines IL-6, CXCL-8 (IL-8), CXCL10 (IP-10, interferon-inducible protein 10), CCL11 (eotaxin-1), and CXCL9 (MIG, monokine induced by IFNγ) using a cytometric bead array and FCAP Array Software (BD Biosciences) and flow cytometer (Facscalibur, BD Pharmigen).

6.3.9 Immunohistochemistry for alpha-smooth muscle actin (α-SMA)

Immunohistochemistry was carried out on glycol methacrylate resin-embedded sections of active pemphigoid conjunctiva, uninflamed pemphigoid and normal conjunctiva, prepared as described in **Chapter 4, section 4.3.2**. The staining procedure was identical apart from the primary antibody being a monoclonal mouse antibody against human alpha-smooth muscle actin (M0851 Dako, Cambridgeshire UK), diluted 1:10, and the secondary antibody being biotinylated rabbit anti-mouse immunoglobulin (Dako) diluted 1:200. Vascular smooth muscle in the sections was used as a positive control, and the two negative controls used were absence of the primary antibody and an isotype-matched irrelevant monoclonal antibody (mouse IgG2a, Dako), diluted 1:10. The number of stromal cells stained with alpha-smooth muscle actin, were counted in a masked fashion in 5 representative high power fields per patient, using an Olympus BX51 microscope with image analysis software.

6.3.10 Immunodetection of myofibroblast differentiation in mechanically stressed fibroblast-seeded collagen lattices

Mechanically stressed fibroblast-seeded collagen lattices were prepared to assess for myofibroblast differentiation (Garrett *et al.*, 2004). To generate mechanical

stress in the collagen matrix, after polymerization of the lattice mixture, the lattices remained attached to the culture well and were cultured in FCM for 24hours. At 24hours, the lattice was fixed with 4% paraformaldehyde, then gently detached completely with a pipette tip. Triplicate samples per donor were tested, and at least 6 donors were tested in each of the groups: active pemphigoid fibroblasts, uninflamed pemphigoid fibroblasts, normal conjunctival fibroblasts. The fixed collagen lattices were permeabilised for 15 minutes with 1% Triton X-100 (Sigma-Aldrich) at room temperature (RT). For immunostaining of α-SMA, monoclonal anti-α-SMA antibody conjugated with Cy3 (Clone 1A4; Sigma-Aldrich) was added to the lattices (1:200 in blocking buffer) for 3 hours at RT. After the lattices were rinsed with PBS and exposed for 1 minute to 4',6-diaminidino-2-phenylindole (DAPI) dye (1:5000 in PBS; Sigma-Aldrich), they were rinsed and mounted on a 35mm MatTek glass-bottomed dish (MatTek Corporation, Ashland, MA) with a fluorescent mountant and covered with a coverslip. Cells were viewed with a laser scanning confocal microscope (Leica TCS_SP2, Milton Keynes UK). The proportion of myofibroblasts and fibroblasts was determined by counting the number of cells containing assembled α-SMA stress fibres as a proportion of the total number of cells visualized by DAPI-stained nuclei. Five random fields of view at x40 final magnification were analysed with Leica image analysis software.

6.3.11 Surface expression of activation markers and costimulatory molecules by early passage pemphigoid fibroblasts and normal conjunctival fibroblasts.

Preliminary experiments to characterize the surface expression of activation markers and costimulatory molecules by early passage pemphigoid versus normal fibroblasts were attempted. Passage 0 fibroblasts were obtained directly from the primary biopsies using trypsin/EDTA, in a similar manner to that described in **chapter 4, section 4.3.3**. As there was no reliable fibroblast marker, passage 0 fibroblasts for these experiments were not obtained until at least 6 weeks in culture, to allow a sufficient time for any potential contaminating epithelial cells to die. Passage 1 fibroblasts were obtained from the 6 well-plate cultures which were

necessary to grow sufficient numbers of passage 1 cells for further passaging into flasks (as described in **chapter 4, section 4.3.3**). Three-colour immunofluorescence staining with either anti-ICAM-1^{PE}, anti-CD80^{FITC}and anti-HLA-DR^{PerCP}, or anti-CD154^{FITC}, anti-CD86^{PE}, and anti-CD40^{PECy5} was conducted as described in **chapter 4, section 4.3.8**.

6.3.12 Statistical Analysis

Differences between 2 groups were examined for statistical significance using the Mann-Whitney U test and one-way analysis of variance (ANOVA) was used to compare the means of 3 or more unmatched groups. $P < 0.05$ was considered significant.

6.4 Results

6.4.1 Pemphigoid and normal conjunctival fibroblasts appear to have similar morphology

No clear differences in the morphology of pemphigoid fibroblasts compared with normal conjunctival fibroblasts were detected (**Figure 6.1**), nor between pemphigoid fibroblasts obtained from acutely inflamed tissue compared with chronic uninflamed tissue.

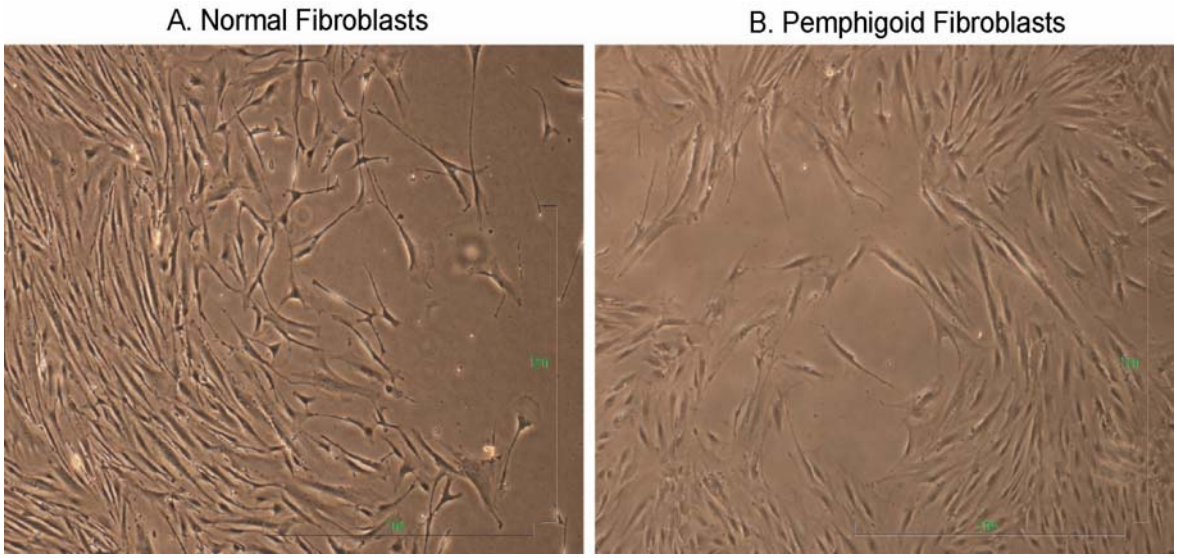

Figure 6.1 Morphology of normal and pemphigoid fibroblasts *in vitro*. Inverted phase contrast microscopy of A. normal conjunctival fibroblasts 6 days after the 4th subpassage and B. pemphigoid conjunctival fibroblasts from acutely inflamed tissue 5 days after the 4th subpassage. Pemphigoid fibroblasts were similar in appearance to normal conjunctival fibroblasts. Magnification x100, bar= 1mm.

6.4.2 Comparison of early versus late passage pemphigoid fibroblasts, and actively inflamed versus uninflamed pemphigoid fibroblasts

Comparison of early versus late passage normal control conjunctival fibroblasts and pemphigoid conjunctival fibroblasts showed no significant differences in all assays including collagen contraction (**Figure 6.2**), proliferation and migration. Comparison of actively inflamed versus uninflamed pemphigoid conjunctival fibroblasts in all assays did not show any clinically or statistically significant differences (**Figure 6.3**).

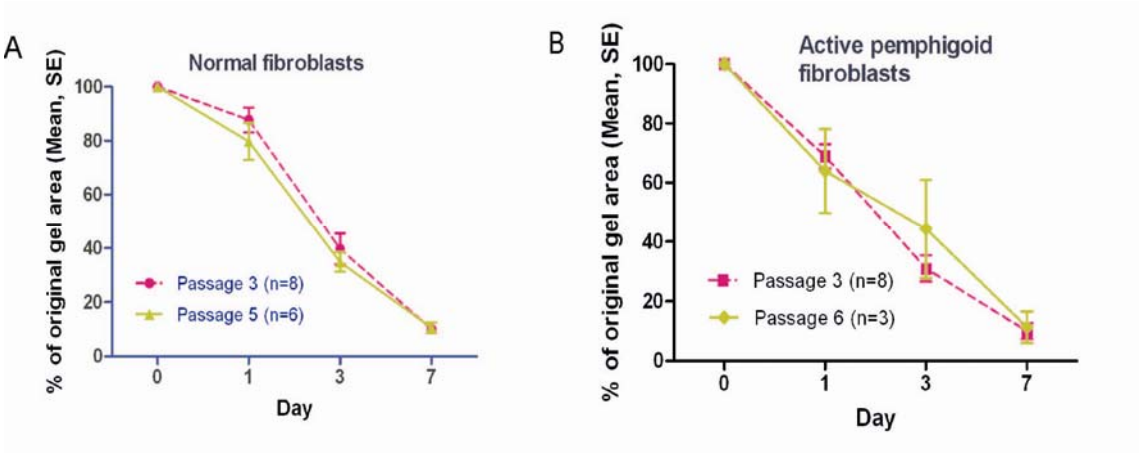

Figure 6.2 Comparison of collagen contraction by early versus late passage fibroblasts. A. No difference in collagen contraction over 7 days in FCM between passage 3 versus passage 5 normal conjunctival fibroblasts. **B.** No difference in collagen contraction over 7 days in FCM between passage 3 and passage 6 actively inflamed pemphigoid conjunctival fibroblasts. n= number of individual experiments.

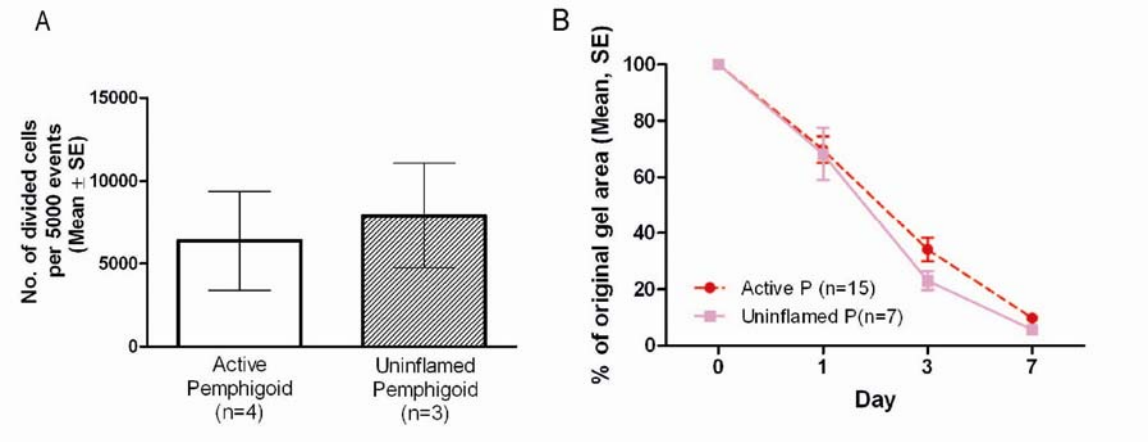

Figure 6.3 Comparison of actively inflamed versus uninflamed pemphigoid conjunctival fibroblasts. A. No difference in number of divided cells between actively inflamed versus uninflamed pemphigoid fibroblasts after 96 hours culture in FCM. n= number of individual donors. **B.** No clinically significant difference in collagen contraction over 7 days in FCM, between actively inflamed versus uninflamed pemphigoid fibroblasts. Although there appears to be a difference in the gel areas at day 3, the magnitude of this difference is small (34% - 23% = 11%) and not considered clinically significant. P = pemphigoid, n= number of individual experiments

6.4.3 Pemphigoid fibroblasts have a greater number of cell divisions than normal conjunctival fibroblasts

Pemphigoid conjunctival fibroblasts showed significantly increased numbers of divided cells compared with normal conjunctival fibroblasts after 96 hours in culture (average 7026 ± 2020 vs 1216 ± 558 divided cells per 5000 events, P =0.01, **Figure 6.4**.)

Interestingly, despite the increased number of cell divisions detected by flow cytometry, in practical terms when passaging and growing pemphigoid fibroblasts, a confluent flask of pemphigoid fibroblasts more often than not would yield fewer viable cells on a cell count compared with a same-size same-confluence flask of normal control fibroblasts. This could have been due to more rapid cell death of pemphigoid fibroblasts in response to trypsinisation, or due to a larger pemphigoid cell size (although the latter appeared to be less likely, based on subjective (non-quantitative) morphology comparisons).

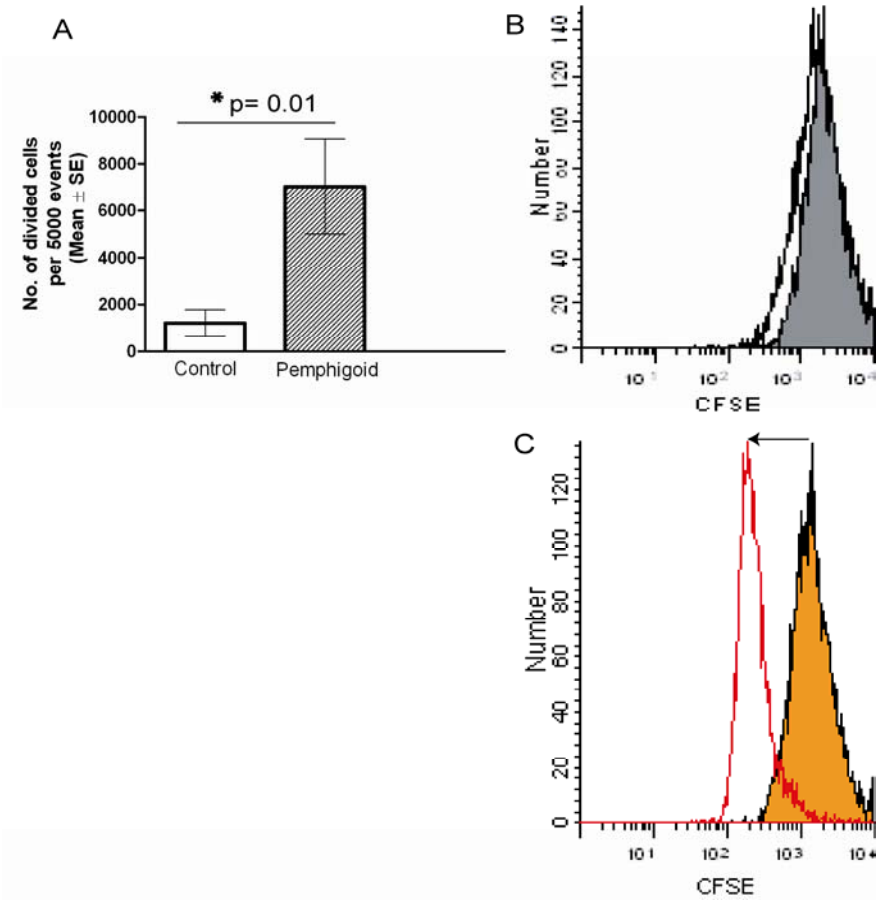

Figure 6.4 Fibroblast cell division assessed by flow cytometric measurement of dilution of the fluorescent label CFSE (carboxyfluorescein diacetate, succinimidyl ester). CFSE is bound to proteins in the cell. For each division, the amount of CFSE in each daughter cell is half that of the parent. **A.** After a 96-h culture period in 10% serum-containing medium, pemphigoid fibroblasts had significantly increased numbers of divided cells compared with normal control conjunctival fibroblasts (P = 0.01). The ratio of divided cells was calculated by measuring the number of cells with a diluted CFSE concentration per 5000 undivided cells. Results are the mean and SE from 5 or more individuals in each group. **B.** Representative histogram of number of events (y axis) versus CFSE fluorescence (x axis) in a normal fibroblast line. Cell division is indicated by decreased fluorescence to the left of the time 0 peak. Grey filled-in peak is the fluorescence of CFSE in the cells at time 0. Black line is the fluorescence at 96-h. **C.** Histogram of number of events versus CFSE fluorescence in a pemphigoid fibroblast line. Orange filled-in peak is the fluorescence of CFSE in the cells at time 0. Red line is the fluorescence at 96-h. There are more divided cells in the pemphigoid fibroblasts compared with the normal fibroblasts. There is only one peak of divided cells, suggesting that the size of the well limited further cell division.

6.4.4 Increased migration by pemphigoid conjunctival fibroblasts under serum-free conditions

The number of pemphigoid fibroblasts that migrated through the porous membranes towards the 0.1% bovine serum albumin serum-free medium (SFM) was significantly higher than the number of normal control fibroblasts that migrated towards SFM (72 ± 18 versus 33 ± 11, *P* = 0.04) (Figure 4). No significant difference between pemphigoid and normal fibroblasts in the number of cells migrating towards 10% serum-containing FCM was detected (data not shown).

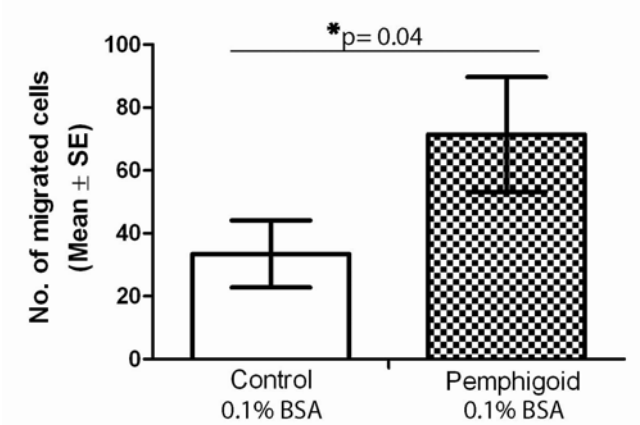

Figure 6.5 Comparison of pemphigoid versus normal conjunctival fibroblast migration towards 0.1% bovine serum albumin (BSA) serum-free medium (SFM). Conjunctival fibroblasts were seeded in the upper chambers of porous membranes and allowed to migrate overnight towards the lower chamber containing the test substance, in this case 0.1% bovine serum albumin (BSA) serum-free medium (SFM). Pemphigoid fibroblasts migrated more than normal control fibroblasts under serum-free conditions. Results are the mean and SE of 8 individual experiments. *P = 0.04.

6.4.5 Pemphigoid fibroblasts show increased collagen contraction in response to tumour necrosis factor-α, and reduced contraction in response to interleukin-13, compared with normal conjunctival fibroblasts

Collagen contractility of the fibroblasts was next assessed. Although there was no detectable difference in the contraction of relaxed collagen gel lattices by pemphigoid and normal conjunctival fibroblasts in either serum-free medium (SFM) (**Figure 6.6 A**) or 10% serum-containing fibroblast culture medium (FCM) (**Figure 6.6 B**), the pemphigoid fibroblasts did contract collagen in response to tumour necrosis factor-α (TNFα) (**Figure 6.6 C**). In comparison, the findings in chapter 4 showed that normal conjunctival fibroblasts do not contract collagen in response to TNFα (see **Chapter 4, section 4.4.2**). In contrast, the addition of interleukin-13 (IL-13) to the collagen lattice conditioned medium resulted in less contraction by pemphigoid fibroblasts compared with normal fibroblasts, relative to the amount of contraction in the SFM negative control (**Figure 6.6 D**).

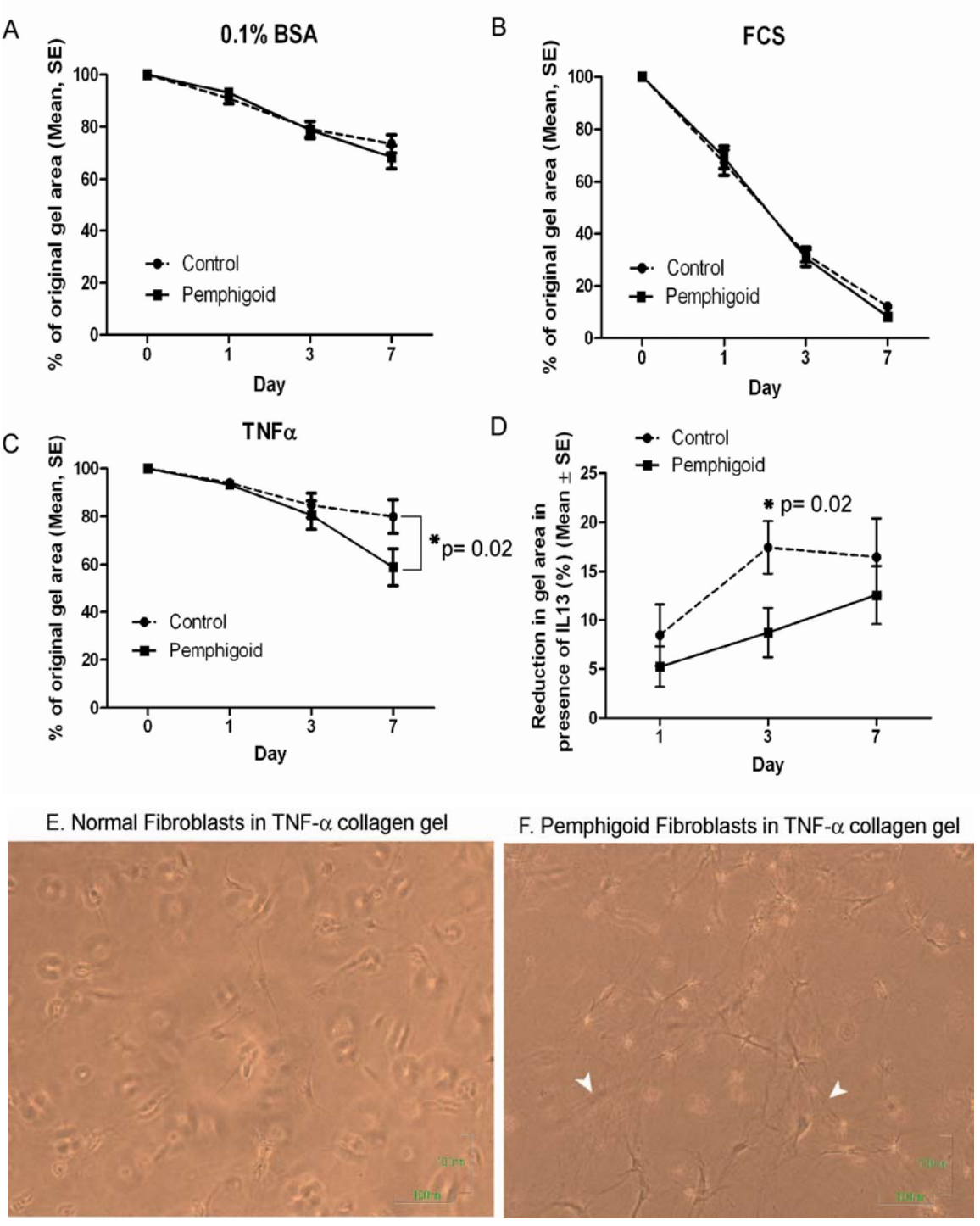

Figure 6.6 Fibroblast-populated collagen gel lattice contraction over 7 days by pemphigoid versus normal conjunctival fibroblasts showing **A.** no difference in contraction in 0.1% bovine serum albumin (BSA) serum-free medium and **B.** no difference in contraction in 10% foetal calf serum (FCS)-containing medium. C. In the presence of 10ng/ml tumour necrosis factor-α (TNFα),

pemphigoid fibroblasts contract collagen more than normal fibroblasts by day 7 (*P* = 0.02). Differences in cell morphology in the TNFα gel observable at day 7 are shown below in **E** and **F. D.** In the other hand, in the presence of interleukin-13 (IL-13), at day 3 pemphigoid fibroblasts contract collagen less vigorously than normal fibroblasts do at day 3 (*P* = 0.02), but by day 7 there is no significant difference. The reduction in gel area in response to IL-13 at day 3 is less in pemphigoid fibroblasts than normal fibroblasts (relative to the serum-free medium negative control). Results of all graphs are the mean and SE from 6 or more donors per group. **E.** Morphology of normal fibroblasts at day 7 in a collagen lattice treated with TNFα. The cells suspended in the collagen matrix have elegantly curved cell processes and there is no evidence of contractile bands of collagen. **F.** Morphology of pemphigoid fibroblasts at day 7 in a collagen lattice treated with TNFα. Fibroblast cell processes are straighter, shorter and thicker, and there are contractile bands of collagen radiating from the cell processes (arrowheads). Magnification x 200. Bar = 100nm.

6.4.6 Pemphigoid fibroblasts secrete more matrix metalloproteinase-3 (mmp-3) and less mmp-13 in the presence of serum

During day 0-3 of collagen lattice contraction in 10% serum-containing medium, pemphigoid fibroblasts secrete more matrix metalloproteinase-3 (mmp-3) (P =0.04) and less mmp-13 (P =0.02) compared with normal conjunctival fibroblasts (**Figure 6.7 A**). During day 3-7 of collagen lattice contraction in 10% serum-containing medium, there was no difference in mmp and tissue inhibitor of matrix metalloproteinase (timp) secretion between pemphigoid and normal fibroblasts (data not shown). In the presence of IL-13, both normal and pemphigoid fibroblasts secrete less mmp-3 (**Figure 6.7 B,C**), and when pemphigoid fibroblasts and normal fibroblasts are compared directly, pemphigoid fibroblasts secrete less mmp-8 than normal fibroblasts in the presence of IL-13 (**Figure 6.7 D**). An increase in mmp-3 secretion by pemphigoid fibroblasts was also observed during collagen contraction in serum-free medium from day 0-7(data not shown).

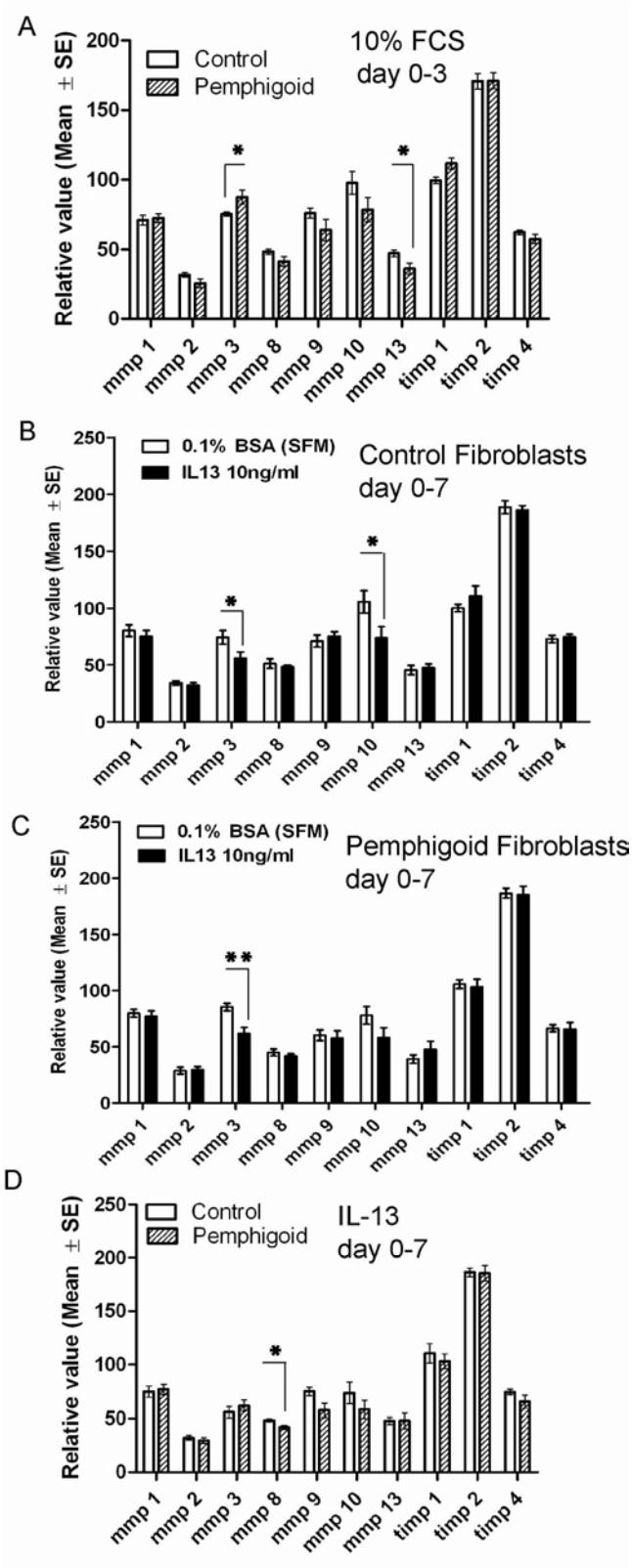
A
Relative value (Mean ± SE)
Control
Pemphigoid
10% FCS
day 0-3
mmp 1
mmp 2
mmp 3
mmp 8
mmp 9
mmp 10
mmp 13
timp 1
timp 2
timp 4
B
0.1% BSA (SFM)
IL13 10ng/ml
Control Fibroblasts
day 0-7
C
Pemphigoid Fibroblasts
day 0-7
D
IL-13
day 0-7

Figure 6.7. Matrix metalloproteinase (mmp) and tissue inhibitor of matrix metalloproteinase (timp) levels in conditioned medium of conjunctival fibroblast-populated collagen gels, detected by antibody-coated membrane array. Data are relative values compared with the membrane array negative control. **A.** During day 0-3 of collagen lattice contraction in 10% foetal calf serum (FCS)-containing medium, pemphigoid fibroblasts secreted higher levels of mmp-3 (P =0.04) and lower levels of mmp-13 (P = 0.02) compared with normal fibroblasts. **B.** During day 0-7 of collagen lattice contraction in the presence of IL-13 10ng/ml in SFM, normal conjunctival fibroblasts secrete lower levels of mmp-3 (P =0.04) and mmp-10 (P =0.01), and **C.** pemphigoid fibroblasts similarly secrete lower levels of mmp-3 (P =0.004). **D.** When compared directly in the presence of IL-13, pemphigoid fibroblasts secrete less mmp-8 (P =0.02) compared with normal fibroblasts. Results are the mean and SE from 6 individual donors per graph. *P < 0.05 **P < 0.01

6.4.7 Pemphigoid fibroblasts secrete more type I collagen.

During day 3-7 of collagen lattice contraction in 10% serum-containing medium, pemphigoid fibroblasts secrete more C-terminal propeptide of type I collagen (CICP) compared with normal conjunctival fibroblasts (median 62.2 versus 31.4 ng/ml, *P* = 0.03) (**Figure 6.8**).

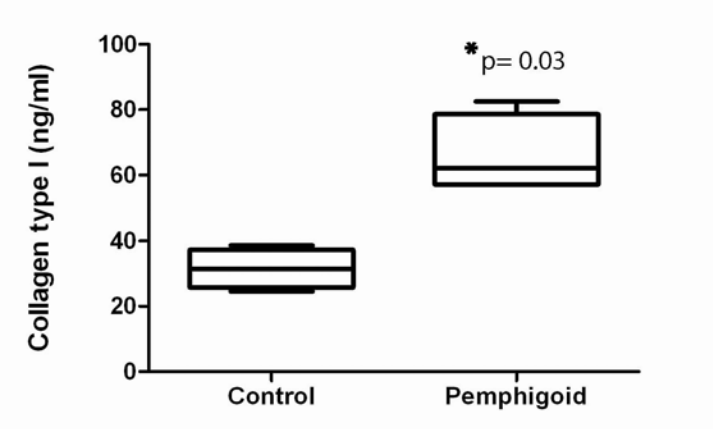

Figure 6.8 Comparison of C-terminal propeptide of type I collagen (CICP) secretion by pemphigoid fibroblasts versus normal conjunctival fibroblasts during day 3-7 of collagen lattice contraction in 10% serum-containing medium. Pemphigoid fibroblasts secrete more type I collagen than normal fibroblasts (*P* =0.03). Results are from 4 individual donors in box-whisker plots showing median, upper and lower quartiles, maximum and minimum.

6.4.8 Pemphigoid fibroblasts secrete more CCL11 (eotaxin-1) in response to IL-13.

Pemphigoid fibroblasts secreted more CCL11 (eotaxin-1) in response to IL-13 (**Figure 6.9**). No significant differences between pemphigoid and normal fibroblasts with respect to secretion of IL-6, CXCL8 (IL-8), CXCL10 (IP-10) or CXCL9 (MIG) were detected, either in the absence of cytokines or in response to stimulation by IL-4, IFNγ or TGF-β.

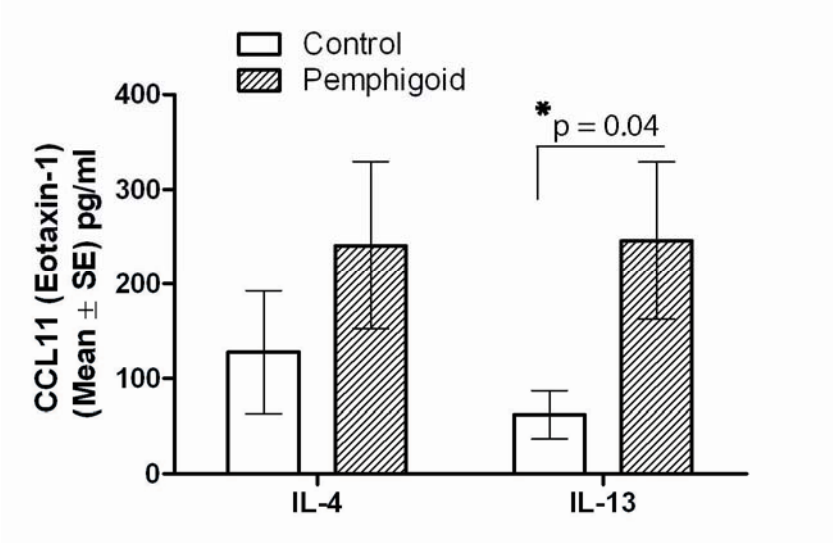

Figure 6.9 Comparison of CCL11 (eotaxin-1) secretion by pemphigoid fibroblasts versus normal fibroblasts in response to IL-4 and IL-13. Pemphigoid fibroblasts secrete more CCL11 in response to IL-13 than normal fibroblasts (*P* =0.04). Results are the mean and SE from at least 6 individual donors.

6.4.9 Myofibroblast differentiation

There was very little stromal α-SMA staining in both pemphigoid and normal bulbar conjunctiva, and no differences were detected between pemphigoid and normal conjunctiva. No significant differences were detected between pemphigoid fibroblasts and normal fibroblasts, regarding the expression of assembled α-SMA filaments in collagen lattices after 24 hours of mechanical stress (data not shown).

6.4.10 Surface expression of activation markers by early passage pemphigoid fibroblasts versus normal conjunctival fibroblasts

It was not possible to obtain sufficient numbers of passage 0 cells for flow cytometry. Results of preliminary experiments using passage 1 cells from two normal control fibroblast cell lines, one active pemphigoid cell line and one uninflamed pemphigoid cell line, are shown in **Figure 6.10**. These experiments need to be repeated before any conclusions can be made, but the findings suggest that there does not appear to be any difference in HLA-DR or costimulatory molecule expression, between early passage pemphigoid versus normal conjunctival fibroblasts. The active pemphigoid fibroblast cell line tested appeared to have reduced ICAM expression compared to the other cell lines.

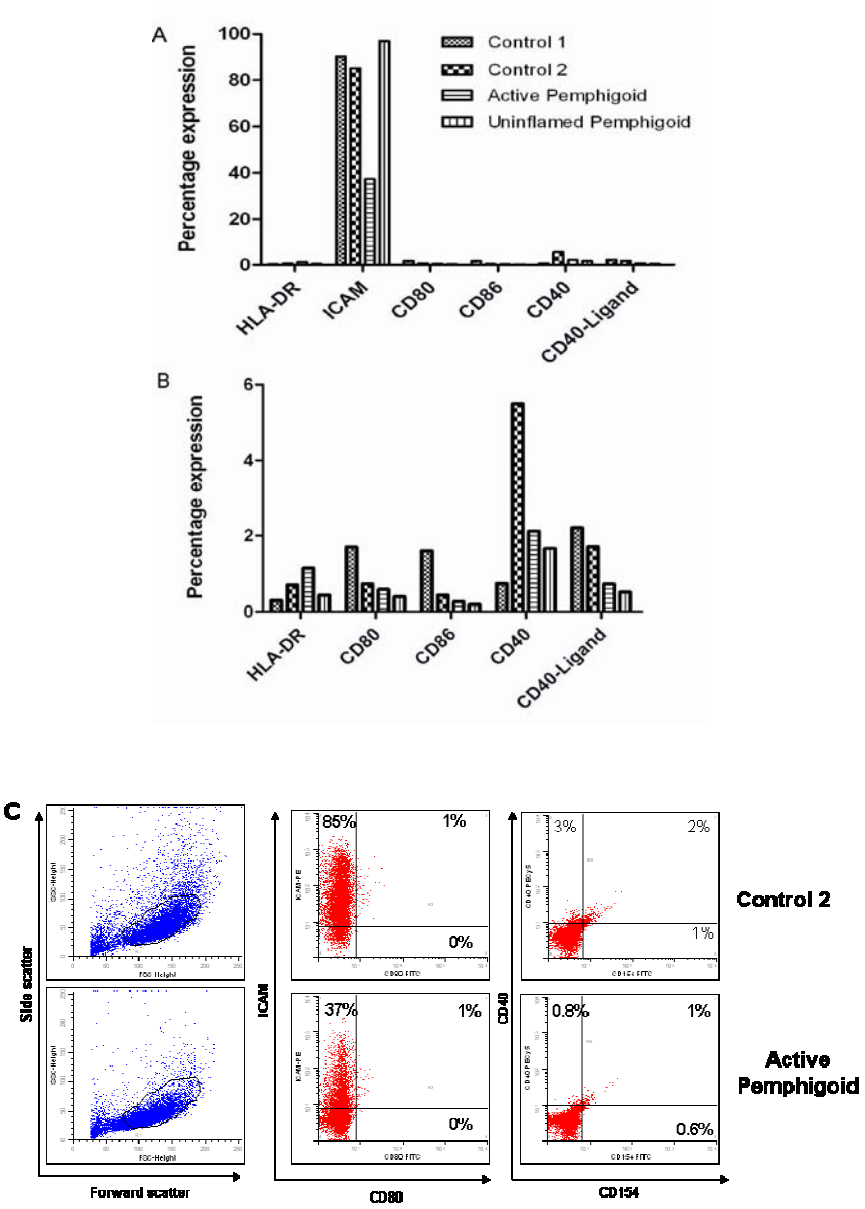

Figure 6.10 Expression of surface markers of activation and costimulatory molecules by passage 1 pemphigoid and normal conjunctival fibroblasts. Passage 1 cells from two normal, one active pemphigoid and uninflamed pemphigoid conjunctival fibroblast cell lines were tested. **A.** The percentage of conjunctival fibroblasts expressing on their cell surface HLA-DR, ICAM, CD80, CD86, CD40, and CD154 was determined by flow cytometry. High ICAM expression was detected in all cell lines except the active pemphigoid cells. **B.** shows a magnified view of surface expression of the other molecules apart from ICAM. There was negligible expression of HLA-DR and costimulatory molecules apart from 5.5% CD40 expression by the control 2 cell line. **C.** shows representative density plots of the control 2 and active pemphigoid cell line.

6.5 Discussion

The results in this chapter show that conjunctival fibroblasts from ocular mucous membrane pemphigoid (ocular MMP) patients display a distinct fibrotic phenotype characterized by increased cell division, chemotaxis, collagen and matrix metalloproteinase synthesis, that is maintained over multiple passages *in vitro*. In the presence of the pro-fibrotic type 2 helper T cell mediator interleukin-13 (IL-13) which was shown to be expressed in ocular MMP in chapter 5, pemphigoid fibroblasts appear to respond with less contraction and reduced matrix metalloproteinase production, but with increased secretion of the chemokine CCL11 (eotaxin-1). We did not detect any differences between pemphigoid fibroblasts isolated from acutely inflamed versus uninflamed tissue.

Increased proliferation of ocular MMP conjunctival fibroblasts has previously been observed (Roat *et al.*, 1989), as has increased expression of macrophage migration inhibitory factor, macrophage-colony stimulating factor, connective tissue growth factor and heat shock protein 47 by ocular MMP fibroblasts (Razzaque *et al.*, 2003b; Razzaque *et al.*, 2004). Preliminary studies have also suggested that ocular MMP fibroblasts express the proto-oncogene c-myc (Hunt LE, et al. IOVS 1991;32:ARVO Abstract 938) and show ultrastructural changes indicating increased protein synthesis which persist in culture (Biesman BS et al. IOVS 1994;Suppl 35(4):ARVO Abstract 170). The findings in this chapter of increased proliferative, secretory and matrix synthetic activity by pemphigoid fibroblasts are in agreement with these previous reports.

Unlike the findings in scleroderma however, no difference in myofibroblast differentiation by pemphigoid fibroblasts and tissues compared to normal cells and tissues was able to be detected. Simple cytoplasmic expression of unassembled actin alone is not an indicator of myofibroblast differentiation; morphologic detection of α-smooth muscle actin (α-SMA) filaments assembled as stress fibres

by confocal microscopy is necessary (Tomasek *et al.*, 2002). It is possible that 24hrs duration of mechanical stress was too short to enable differences between pemphigoid and normal fibroblasts to emerge, and perhaps 48 hours might have allowed differences to have been observed. The immunohistochemical staining of normal versus pemphigoid conjunctival tissue needs to be repeated using an alternative α-SMA antibody, and the mechanically stressed gel experiments also need to be repeated using different durations of mechanical stress and/ or in the presence of TGFβ, which promotes myofibroblast differentiation particularly in the presence of mechanical tension (Vaughan *et al.*, 2000). It is also possible that cell types other than the resident tissue fibroblasts are the source of myofibroblasts in ocular MMP. There is emerging evidence from studies of pulmonary fibrosis and other disorders that epithelial cells could be an important source of myofibroblasts, via the process of epithelial-mesenchymal transdifferentiation (EMT) (Radisky & LaBarge, 2008).

Increased matrix metalloproteinase-3 (mmp-3) and decreased mmp-13 secretion by pemphigoid conjunctival fibroblasts during collagen lattice contraction was observed in this study. Mmp-3, also known as stromelysin-1, degrades pro-mmps-1,-7,-8,-9,-13, proteoglycans, laminin and fibronectin. It has been found to play a role in skin and corneal wound healing and pterygia (Daniels *et al.*, 2003b; Li *et al.*, 2001; Wong *et al.*, 2002), and is produced during collagen lattice contraction by human Tenon's capsule fibroblasts (Daniels *et al.*, 2003a). Broad spectrum inhibition of mmp activity has reduced matrix contraction and collagen production in vitro (Daniels *et al.*, 2003a) and in an animal model of glaucoma surgery . On the other hand, the profibrotic mediator IL-13, which was found to be present in the stromal tissues of ocular MMP in chapter 5, causes a reduction in mmp-3 synthesis by both normal and pemphigoid conjunctival fibroblasts, so whether the effect of IL-13 overrides the inherent behaviour of pemphigoid fibroblasts with regard to mmp-3 synthesis *in vivo* is yet to be determined. Mmp-13 (collagenase-3) digests collagens I,II, II,IV, gelatin, fibronectin, and proteoglycans. Reduced mmp-13 could

either result in a net increase in matrix due to reduced matrix degradation, or could be associated with reduced mmp-dependent fibroblast locomotion through the extracellular matrix and subsequent matrix contraction (Daniels *et al.*, 2003a). Which of these two alternatives is the dominant process *in vivo* is currently uncertain.

Characteristics of fibrotic fibroblasts are probably unique to each disease, given that, for example, migration can be increased or decreased, depending on the disease from which the fibroblasts have been isolated (Rieder *et al.*, 2007). There is also evidence that the phenotype of fibrotic fibroblasts varies according to disease duration and severity (Corriveau *et al.*, 2008).

Developing effective antifibrotic therapies will require understanding of both the cellular sources of the profibrotic fibroblasts, and the mechanisms that activate and recruit these cells to sites of scarring. In order to elucidate what happens in ocular MMP, knowledge of what happens in a disease such as scleroderma provides framework from which to work, with the understanding that although the processes occurring in the two diseases are clearly not identical, there are some definite similarities. In scleroderma, there appears to be a process of selection leading to the propagation of certain apoptosis-resistant profibrotic subpopulations of fibroblasts, which are preferentially expanded or selectively activated within lesional tissue (Abraham *et al.*, 2007). Moreover, it appears that both resident and circulating cell types can contribute to fibroblast differentiation, thus in part accounting for the observed heterogeneity of fibroblasts both derived from within and between tissues. Potential sources of profibrotic fibroblasts include resident mesenchymal cells, bone-marrow-derived mesenchymal precursors (Hashimoto *et al.*, 2004), circulating fibroblast progenitors including peripheral blood mononuclear cells (Abraham *et al.*, 2007), epithelial-mesenchymal transition (EMT), and pericytes. Apart from selective amplification of subpopulations of activated phenotype fibroblasts derived from various sources, induction of the profibrotic

fibroblast phenotype is also thought to be influenced by profibrotic mediators in the inflammatory milieu (Abraham *et al.*, 2007; Karlson *et al.*, 2006), and sometimes cell-cell contact between immune cells and fibroblasts (Trojanowska, 2004). The phenotypic changes have been shown, in pulmonary fibrosis fibroblasts, to reflect genome-wide derangements of the gene expression pathway (Larsson *et al.*, 2008).

In ocular MMP, there is significant inflammation induced by the autoimmune process which could selectively amplify resident, bone-marrow derived or circulating mesenchymal cells influenced by the inflammatory milieu (Bernauer *et al.*, 1993b). Furthermore, TGFβ-driven epithelial-mesenchymal transition (EMT) could also play a role, given that when conjunctival epithelial injury is incurred in the absence of treatment with systemic immunosuppression, rapid worsening of ocular MMP is observed (Mondino *et al.*, 1979).

6.6 Conclusion

Conjunctival fibroblasts from ocular MMP patients appear to have a profibrotic phenotype, which is maintained *in vitro*. This indicates that the mechanisms regulating fibrosis in ocular MMP are distinct from those controlling inflammation. It is likely that immunosuppressive therapy targeting inflammation is ineffective at completely arresting fibrosis in ocular MMP because of this dissociation of inflammation from fibrogenesis. Fibroblasts from actively inflamed ocular MMP tissue do not appear to differ significantly from fibroblasts derived from uninflamed tissue. This suggests that the altered phenotype may be induced at the initiation of the conjunctival scarring pathway, and does not vary according to the current inflammatory milieu. The altered phenotype may be dependent on the severity of scarring and disease duration rather than on the current inflammatory milieu.

Chapter 7

Conclusions

7.1 Significance

There are a number of key findings from this work. Although there is clear evidence that acute inflammation following conjunctival injury in ocular MMP, either iatrogenic due to surgery or due to the autoimmune process, triggers rapid and progressive fibrosis, conjunctival fibrosis in ocular MMP can still progress despite apparent clinical control of inflammation by treatment with immunosuppressive therapy. This echoes the situation in many fibrotic disorders where immunosuppressive therapy affords limited benefit in attenuating fibrosis.

Administering higher doses of systemic immunosuppression in the form of pulse intravenous methylprednisolone as adjunctive therapy to patients receiving high dose oral cyclophosphamide and corticosteroids cannot be recommended on the basis of the findings in this study. Although relatively well tolerated, it does not induce more rapid control of inflammation, and can lead to bone loss.

Discovering the lack of effect of pulse intravenous methylprednisolone provides a stimulus to assessing alternative therapies for treating recalcitrant inflammation in ocular MMP, such as TNFα antagonists. Such therapy might be expected to have an effect in up to 90% of patients with active ocular MMP, given the observed frequency of tissue expression of TNFα in these patients. The findings of modest pro-fibrotic effects of TNFα on conjunctival fibroblasts *in vitro* are reassuring in regard to the possible anti-fibrotic, rather than profibrotic, effects of TNFα antagonists. The persistently elevated expression of TNFα in clinically uninflamed conjunctiva is in keeping with previous descriptions of ‘white inflammation’, which may be driving progressive fibrosis. Administering higher doses of systemic immunosuppression might possibly control this underlying residual inflammation, however the majority of mucous membrane pemphigoid patients are elderly and susceptible to toxicity resulting from high dose immunosuppression; furthermore, some of the patients in whom underlying residual inflammation was detected were

already on maximal tolerated immunosuppression (including cyclophosphamide) when the clinically uninflamed biopsy was taken. Finding a suitable local anti-inflammatory, anti-fibrotic therapy as an adjunct to maximal dose- minimal adverse effect-systemic immunosuppression would be ideal.

The modest profibrotic effects of TNFα and prominence of T cells in the ocular MMP substantia propria led to work investigating the key pro-fibrotic T cell-derived cytokine IL13. The expression of IL-13 in ocular MMP indicates a role for Th 2 cells in fibrosis in ocular MMP. The persistent elevated expression of IL-13 in clinically uninflamed conjunctiva suggests that IL-13 has direct profibrotic effects on conjunctival fibroblasts, and that these effects may be enhanced by synergism with other T cell-secreted products.

Finally, although inflammation due to TNFα and IL-13 is likely to play a important role in fibrogenesis in ocular MMP, the question of whether ocular MMP fibroblasts behave autonomously, independent of cytokine influences has been answered. Ocular MMP fibroblasts show a profibrotic phenotype *in vitro* which is clearly different from that of normal conjunctival fibroblasts. Further work investigating the cellular sources of the profibrotic fibroblasts, and the mechanisms that activate and recruit these cells to sites of scarring, is necessary. **Figure 7.1** shows the proposed immunopathogenesis of fibrosis in ocular MMP including the findings from this thesis.

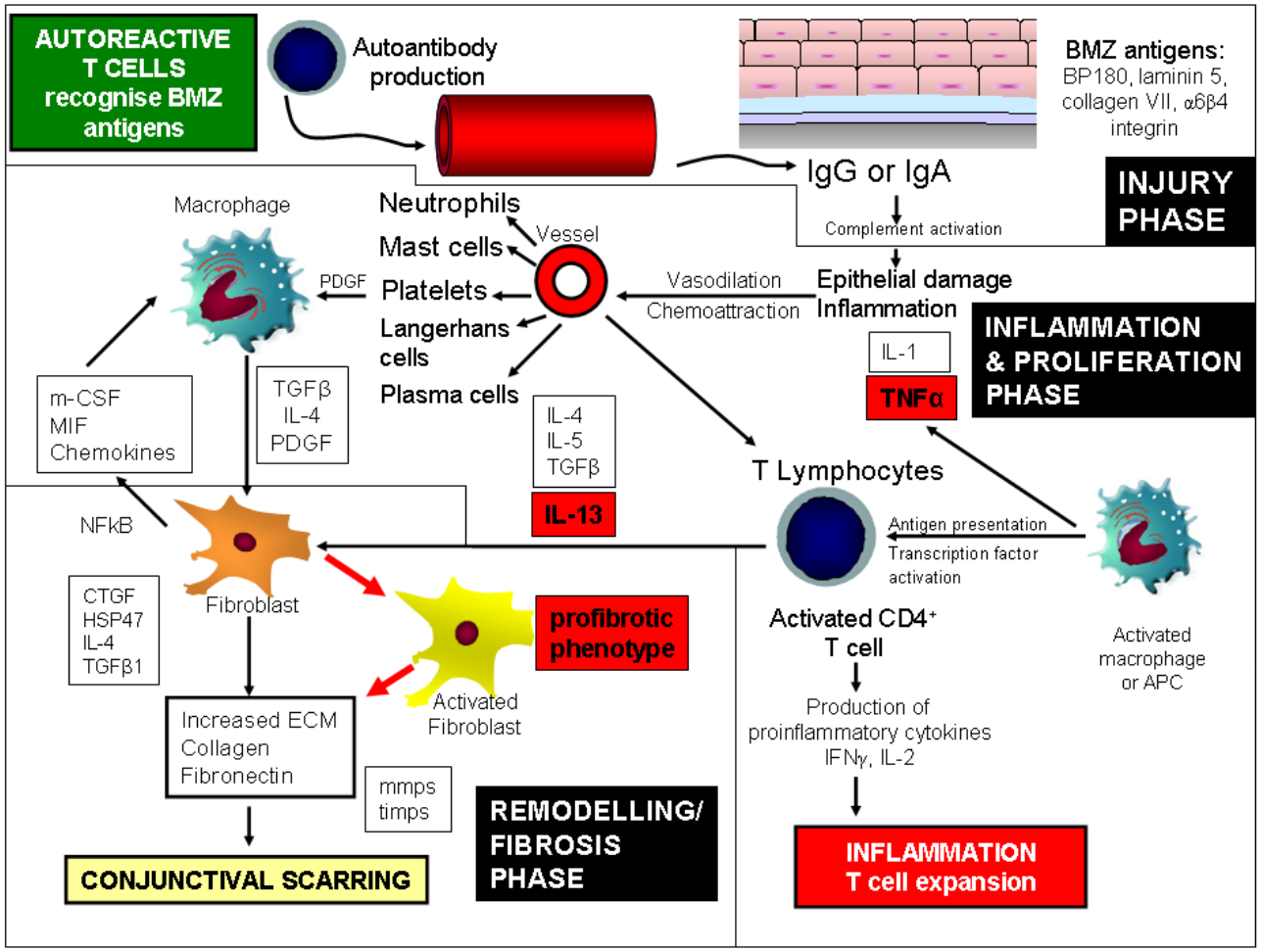

Figure 7.1 Proposed immunopathogenesis of fibrosis in ocular MMP, including findings from this thesis. See next page for detailed legend.TNFα (tumour necrosis factor-alpha), IL-13 (interleukin-13) and conjunctival fibroblasts with a profibrotic phenotype have been shown to be involved.

Legend to Figure 7.1 Proposed immunopathogenesis of fibrosis in ocular MMP.

INJURY PHASE: Autoreactive T cells recognise BMZ (basement membrane zone) antigens (BP180 bullous pemphigoid180kDa antigen, laminin 5, collagen VII, α6β4 integrin) causing B cells in germinal centres to produce autoantibodies IgG (immunoglobulin G) and IgA. These bind to the BMZ and initiate a type II cytotoxic hypersensitivity reaction, activating the complement cascade to cause subepithelial bulla formation.

INFLAMMATION AND PROLIFERATION PHASE: Complement-mediated BMZ, epithelial and connective tissue damage cause vasodilation, release of blood cells and plasma proteins into the damaged site and attracts an acute inflammatory cell infiltrate consisting of neutrophils, activated macrophages, mast cells, platelets, Langerhans cells, and lymphocytes, as well as acute inflammatory cytokine IL-1 (interleukin-1) and TNFα (tumour necrosis factor-alpha) production. TNFα has profibrotic and proinflammatory effects on conjunctival fibroblasts. T cell activation and proliferation characteristic of a Th1 (type 1 helper T cell) response occurs, with IFNγ (interferon-gamma) and IL-2 (interleukin-2) production. Th2 (type 2 helper T cell) cytokines IL-4, IL-5 and IL-13 are also synthesised. IL-13 has a strong profibrotic and proinflammatory effect on conjunctival fibroblasts. Macrophages proliferate and play an important role in scar tissue formation, and also contribute to production of the fibrogenic cytokines TGFβ (transforming growth factor-beta) and PDGF (platelet-derived growth factor).

FIBROSIS PHASE: Fibroblasts become activated, proliferate and synthesise increased extracellular matrix, CTGF (connective tissue growth factor), TGFβ and other cytokines. Endothelial cells may proliferate, forming fibrovascular granulation tissue. The scar tissue is then remodelled, becoming less cellular, and the final result is subconjunctival scarring.

Other abbreviations in Figure 1.4: *APC* antigen presenting cell, *m-CSF* macrophage-colony stimulating factor, *MIF* macrophage migration inhibitory factor, *NFkB* nuclear factor-kappa B, *HSP47* heat shock protein 47, *ECM* extracellular matrix, *mmps* matrix metalloproteinases, *timps* tissue inhibitors of matrix metalloproteinases.

Adapted from Elder (Elder, 1997c) and Razzaque (Razzaque *et al.*, 2003a)

7.2 Strengths and limitations of this study

A major strength of this work was the large patient population from which both the clinical studies and the conjunctival biopsies were able to be obtained, and the novel idea of evaluating *in vitro* the consequences of successful systemic immunosuppressive treatment. This provides an indication of the molecular and cellular alterations following treatment, and could potentially indicate protein expression which is unaffected by immunosuppressive treatment.

The main limitation of this work was its broad scope, which did not allow time for more in depth experiments exploring questions raised by the initial findings. Furthermore, the work is limited in terms of the mechanistic insights it can provide, in part because inability to culture T cells hampered investigation of the consequences of T cell-fibroblast co-culture, and also because of my inability to block IL-13 activity, which meant that experiments isolating the effect of IL-13 could not be carried out. Also, inhibiting fibroblast functional activity *in vitro* may not necessarily translate into a clinically significant reduction in scarring, hence the use of an animal model will be necessary in order to elucidate potentially therapeutic avenues.

7.3 Future work

On the clinical front, for treatment of recalcitrantly inflamed ocular MMP, investigating biological agents such as TNFα antagonists and the anti-B cell agent rituximab in initial pilot studies, followed by appropriately powered controlled studies if these are shown to be feasible, are needed. Such studies may be feasible in MMP because the biological agents probably have a more favourable side effect profile than cyclophosphamide, and could be extended to the larger group of patients with less severe disease; currently only 10% of new referrals to our centres have severe enough disease to meet our criteria for the use of cyclophosphamide, whereas about 80% of all referrals will start systemic

immunosuppressive therapy. Our criteria for the use of cyclophosphamide could also be revised, given that more effective control of inflammation and also fibrosis were observed with the use of this agent in the retrospective review (Chapter 2), although it is more toxic. The results of the IVMP trial (Chapter 3) will be of substantial value in the design and planning of studies using biological therapies. These agents may reduce the time to control and prevent fibrosis more effectively than therapy currently on the “top level” of the immunosuppressive stepladder, cyclophosphamide and steroids.

Given that there is not much difference in terms of cost or side effect profile between TNFα antagonists and rituximab, and rituximab will target the immunopathology upstream of TNFα expression in ocular MMP, rituximab is currently the preferred biological treatment for severely inflamed recalcitrant ocular MMP in our centre. A potential next step for us is to collate the data on patients that we know have been treated with rituximab, both within the IVMP trial and those treated by us and others outside a trial setting, and to use this to plan a pilot study of rituximab, providing the data from the case series is promising.

With regard to *in vitro* work, investigating the effect of TNFα antagonists on normal and pemphigoid conjunctival fibroblasts would be of key interest. If TNFα antagonists are anti-fibrotic, as well as being anti-inflammatory, they would be of clear interest for use in preliminary clinical studies of either frequent subconjunctival or topical therapy, used in conjunction with systemic immunosuppression. Topical TNFα antagonists may also be anti-angiogenic, given that they may increase timp-2 and timp-4 levels.

Successful culture of T cells from ocular MMP biopsies would permit experiments further exploring T cell-fibroblast interactions leading to conjunctival scarring. If increased fibroblast activity was discovered upon co-culture with T cells, investigating whether blockade of IL-13 inhibits this effect would affirm a central

role of IL-13 in T cell-prominent conjunctival fibrosis. These experiments would need to be carried out on both normal and pemphigoid fibroblasts. Effective blockade of IL-13 will require investigation and understanding of the IL-13 receptors present on fibroblasts, and this could be established by immunostaining, flow cytometry, and immunohistochemical staining of the tissues. After confirmation of upregulated protein (and mRNA) expression by fibroblasts of the costimulatory molecules CD154, CD40 and CD80 in response to IL-13, investigating whether blockade of these costimulatory molecules inhibits the effect of T cell co-culture on fibroblasts would also be of interest. The selective costimulation inhibitor abatacept (cytotoxic T lymphocyte-associated antigen 4-Ig) binds to CD80 and CD86 on antigen-presenting cells and is approved for the treatment of rheumatoid arthritis.

Investigating the effects of IL-13, TNFα and also TGFβ on pemphigoid fibroblasts (rather than normal fibroblasts) with respect to other fibroblast functions not explored in this study, such as surface expression of HLA-DR, CD80, CD40, CD154 would be of key interest, given the altered phenotype of pemphigoid conjunctival fibroblasts. Repeating the investigations regarding myofibroblast differentiation in ocular MMP would also be important, to confirm whether or not myofibroblasts are involved in MMP conjunctival scarring.

Establishing the existence of profibrotic subpopulations of fibroblasts in ocular MMP is necessary to support the theory of selective amplification of profibrotic subpopulations. Following this, identifying the cellular sources of the profibrotic fibroblasts, by defining the contribution of circulating and resident mesenchymal progenitor populations in the development of disease, and also identifying the factors that activate and recruit these cells to lesions would be relevant.

Developing a suitable animal model of conjunctival fibrosis will enable testing of some of the hypotheses raised by these findings, and guide development of effective antifibrotic therapies. Possible animal models which could be used

include: (1) the neonatal rabbit model described by Roat et al using a monoclonal mouse anti-rabbit BMZ antibody injected either subconjunctivally or intraperitoneally into neonatal rabbits (Roat *et al.*, 1990). A single injection, given either subconjunctivally or intraperitoneally, evaluated to day 9, resulted in acute conjunctival inflammation and conjunctival basement membrane-bound murine antibody. Modifying this model into a chronic inflammatory model using weekly injections for at least 5 weeks could provide a model of chronic anti BMZ-antibody mediated conjunctivitis which would probably exhibit conjunctival fibrosis; (2) modifying a previously described model of conjunctival scarring in the mouse (Reichel *et al.*, 1998). In this model, a single 25µl subconjunctival injection resulted in scar tissue formation by day 14 in BALB/c mice. Modifying this model into a chronic inflammatory model using weekly injections for at least 5 weeks could provide a suitable model to investigate the immunopathogenesis of inflammation-associated fibrosis in the conjunctiva. Other investigators have used similar models, for example weekly intrarectal injection of the irritant TNBS (2,4,6-trinitrobenzene sulfonic acid) in BALB/c mice to investigate inflammation-associated fibrosis in chronic colitis which mimics human inflammatory bowel disease (Fichtner-Feigl *et al.*, 2007). In the latter model, Th1 cytokines were produced in the first 2 weeks, followed by IL-13 at about week 5, which was followed soon thereafter by TGFβ secretion and the occurrence of fibrosis. As the unscarred mouse eye is very small, using a larger animal such as the rabbit might be preferable.

Reaching the endpoint of studies investigating scar tissue formation and its inhibition by a selected therapy can take prolonged periods of time waiting for scar tissue to form, such that these studies may need to be conducted over several years. A disadvantage of this is that during this prolonged period, alternative new molecules and inhibitory therapies may be discovered, which one might then want to similarly investigate, and moreover, understanding of the effect of the therapy under investigation may also change. Establishing sensitive and specific markers

of pro-scarring activity that can be processed relatively rapidly, such as measurement of upregulated mRNA synthesis of pro-scarring molecules by PCR from a conjunctival swab, could aid in more rapid evaluation of outcomes of anti-scarring therapy in animal models and also in patients, and could obviate the need for such trials to be conducted over prolonged durations of time.

In summary, this work has shown that conventional immunosuppressive therapy does not control conjunctival fibrosis in ocular MMP, that adjunctive pulse IVMP in combination with high dose oral corticosteroids and cyclophosphamide probably has no place in clinical therapy, and that both TNFα and IL-13 are involved in the pathogenesis of ocular MMP. This work has also shown that the mechanisms governing fibrosis are, to a degree, independent of inflammation, due to the findings of an altered profibrotic pemphigoid fibroblast phenotype. Together, these findings show benefit future research investigating conjunctival scarring and the search for new therapeutic options to improve the outcomes in ocular MMP.

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Appendix 1: Scientific output arising from this thesis

Published manuscripts (see Appendix 2)

- Saw VPJ, Dart JKG. Ocular mucous membrane pemphigoid- a strategy for diagnosis and management. *The Ocular Surface* 2008;6(3):128-42.
- Saw VPJ, Dart JKG, Rauz S, Ramsay A, Bunce C, Xing C, Maddison PG, Phillips M. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. *Ophthalmology* 2008 Feb;115(2):253-261.
- Book chapter: Saw VPJ, Dart JKG. Management of ocular mucous membrane pemphigoid. In: Essentials in Ophthalmology-Cornea and External Disease. Editors. Reinhard T, Larkin F. Springer-Verlag, Heidelberg, 2008. Chapter 8, p.154-175.

Manuscripts accepted for publication

- Saw VPJ, Dart RJC, Galatowicz G, Daniels JT, Dart JKG, Calder VL. Tumour necrosis factor-α in ocular mucous membrane pemphigoid and its effect on conjunctival fibroblasts. Investigative Ophthalmology and Visual Science.

Conference presentations and Awards

- Saw VPJ, Dart JKG, Rauz S, Ramsay A. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. 37th Royal Australian and New Zealand College of Ophthalmologists Annual Scientific Congress. Hobart, November 2005.
- Saw VPJ, Dart JKG, Rauz S, Ramsay A. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. 25th Moorfields Eye Hospital Alumni Meeting. London, January 2006.

- Saw VPJ, Dart JKG, Rauz S, Ramsay A. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. 9th Bowman Club Meeting. Birmingham, March 2007.
- Saw VPJ, Dart JKG, Rauz S, Ramsay A. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. International Ocular Surface Society Meeting. Fort Lauderdale, May 2007.
- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Interleukin-13 and tumour necrosis factor-alpha in ocular mucous membrane pemphigoid. 98th Oxford Ophthalmological Congress. Oxford, July 2007. **Awarded the Founder's Cup for Best Laboratory Research Presentation.**
- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Tumour necrosis factor-alpha in ocular mucous membrane pemphigoid. 27th Moorfields Eye Hospital Alumni Meeting. London, January 2008.
- Saw VPJ, Dart JKG. Diagnosis and management of ocular mucous membrane pemphigoid. Midlands Ophthalmological Society Meeting. Wolverhampton, February 2008.
- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Interleukin-13 in ocular mucous membrane pemphigoid. 28th Moorfields Eye Hospital Alumni Meeting. London, January 2009.

Conference Posters

- Saw VPJ, Dart JKG, Rauz S, Ramsay A. Immunosuppressive therapy for ocular mucous membrane pemphigoid: strategies and outcomes. Association for Research in Vision and Ophthalmology Annual Meeting. Fort Lauderdale, May 2006.
- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Tumour necrosis factor-alpha and interleukin-13 in ocular mucous membrane pemphigoid. Fort Lauderdale, May 2007.

- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Interleukin-13 and tumour necrosis factor-alpha in ocular mucous membrane pemphigoid. 98th Oxford Ophthalmological Congress. Oxford, July 2007.
- Saw VPJ, Galatowicz G, Dart JKG, Daniels JT, Calder VL. Interleukin-13 in ocular mucous membrane pemphigoid. GTCBio 6th Annual Cytokines and Inflammation Conference, Orlando, January 2008.

Appendix 2: Publications arising from this thesis

Clinical Practice

JOHN E. SUTPHIN, MD, SECTION EDITOR

Ocular Mucous Membrane Pemphigoid: Diagnosis and Management Strategies

VALERIE P.J. SAW, MD, FRANZCO[1,2] AND JOHN K.G. DART, MA, DM, FRCS, FRCOPHTH[1,2]

ABSTRACT **Ocular mucous membrane pemphigoid presents some of the most challenging dilemmas in anterior segment management. Diagnosis is made difficult by the insensitivity of immunopathological investigations and the differential diagnosis of other scarring conjunctival disorders. The management of the associated ocular surface disease involves control of blepharitis, dry eye, filamentary keratitis, keratinization, lid malposition, and persistent epithelial defect, as well as the identification and avoidance of toxicity. Inflammation associated with the underlying disorder demands the use of systemic immunosuppressive therapy in many patients. New biological immunotherapies have been used when conventional immunosuppressive therapies fail. Ophthalmic plastic surgery is essential for the management of lid malposition and corneal exposure. Improving vision may require the use of specialized contact lenses, cataract surgery, and corneal and ocular surface reconstructive surgery. All surgery must be integrated with ocular surface treatment and immunosuppressive treatment to avoid disease exacerbations.**

KEYWORDS **cicatrizing conjunctivitis, mucous membrane pemphigoid, ocular cicatricial pemphigoid, systemic immunosuppression**

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I. INTRODUCTION

Mucous membrane pemphigoid (**MMP**), previously known as cicatricial pemphigoid, is a systemic autoimmune disease characterized by recurrent blistering of mucous membranes and skin and healing with excessive scar tissue formation.[1] Ocular involvement, also known as ocular cicatricial pemphigoid (**OCP**), occurs in 70% of cases, and blindness has been reported to occur in 27%.[2] MMP is an uncommon condition, with an incidence of 1.16 per million population per year.[3] Ocular MMP remains one of the most difficult anterior segment conditions to manage, and appropriate treatment, commenced early, can prevent devastating irreversible blindness. Although the average age of onset of MMP is 65 years, it can occur in children and young adults,[4,5] and it appears to be more aggressive in younger patients.[6]

II. DIAGNOSIS

A. Clinical Findings

Early diagnosis and initiation of appropriate treatment are essential to prevent the sight-threatening complications of MMP. Ocular MMP typically presents with a red eye and persistent conjunctivitis that has not responded to topical therapy, or with recurrent cicatricial entropion and trichiasis, sometimes following surgical repair. It can also present with ptosis. About 10% of patients present with acute conjunctivitis (Figure 1A) and limbitis (Figure 1B) leading to rapidly progressive scarring (Figure 1C) and surface failure (Figures 1C and D). However, the majority of patients present with subacute or low-grade chronic inflammation and slowly progressive scarring.

The earliest clinical sign in patients with subacute disease is medial canthal scarring, with loss of the plica and caruncle (Figure 1E). Medial canthal scarring is usually an early sign of MMP; it is more frequent in MMP than in conjunctival scarring due to other causes. Linear scarring in the marginal sulcus is sometimes present early in the disease (Figure 1F). Other signs, in order of progression, are subepithelial reticular fibrosis (Figure 1G), infiltration of the tarsal and bulbar conjunctiva (Figure 1F-H), shortening of the fornices (Figure 1H), symblepharon (Figure 1I) and cicatricial entropion (Figure 1J) followed by ankyloblepharon

OUTLINE

(Figure 1K) and then, subsequent to scarring of the lacrimal ductules, which usually occurs late in the disease, a totally dry "skin-like" eye (Figure 1L). Because the diagnosis is often delayed, symblepharon and shortened fornices are frequently already present when the disease is first recognized.[7]

Table 1 summarizes conditions that cause conjunctival scarring. Progression of conjunctival scarring, including scarring that is presumed to have progressed because the eyes were previously healthy, or the onset of cicatricial entropion, should alert the clinician to the possibility of MMP. Diagnosis is complicated because chronic conjunctival inflammation that can induce fibrosis can have a number of causes. Table 1A lists the disorders that are associated with conjunctival scarring that is either static, once the underlying disorder has been controlled or the precipitating drug withdrawn, or only very slowly progressive, having no functional significance. A subset of patients with some of these disorders will develop severe progressive scarring similar to that in MMP.

Table 1B lists the disorders that more commonly cause progressive conjunctival scarring. Unilateral progressive scarring is uncommon and can, rarely, be due to conjunctival tumors masquerading as MMP (Figure 2) or to drugs given for the prolonged treatment of unilateral glaucoma or herpetic eye disease. The clinical progression distinguishes MMP from those subsets of the other mucocutaneous diseases that develop ocular disease similar to MMP, ie, the scarring in MMP is rapidly progressive and functionally significant. In these two groups of patients, and in those with drug-induced pemphigoid, immunosuppressive therapy may be necessary to control the underlying pathology that causes progressive scarring.

The conjunctival signs in MMP may be identical to those observed in other mucocutaneous disorders (graft vs host disease, Stevens-Johnson syndrome, lupus erythematosus,[8,9] lichen planus[10]), and other immunobullous disorders (bullous pemphigoid,[11] linear IgA disease, epidermolysis bullosa acquisita, dermatitis herpetiformis), although the conjunctival signs in the majority of patients with the latter disorders are mild. In many of these mucocutaneous and immunobullous disorders, the skin or oral disease precedes the eye disease, so there is rarely confusion about the diagnosis of the ocular manifestations. However, clinicians may not be aware of the potential for progressive conjunctival scarring in a subset of this group, and they may not realize that subgroups of mucosal linear IgA disease and epidermolysis bullosa acquisita are currently recognized as forms of MMP.[1] In Stevens-Johnson syndrome, major exacerbations of conjunctival inflammation can occur many years after the acute disease, leading to a condition indistinguishable from MMP, both in terms of the clinical signs and immunopathology.[12,13]

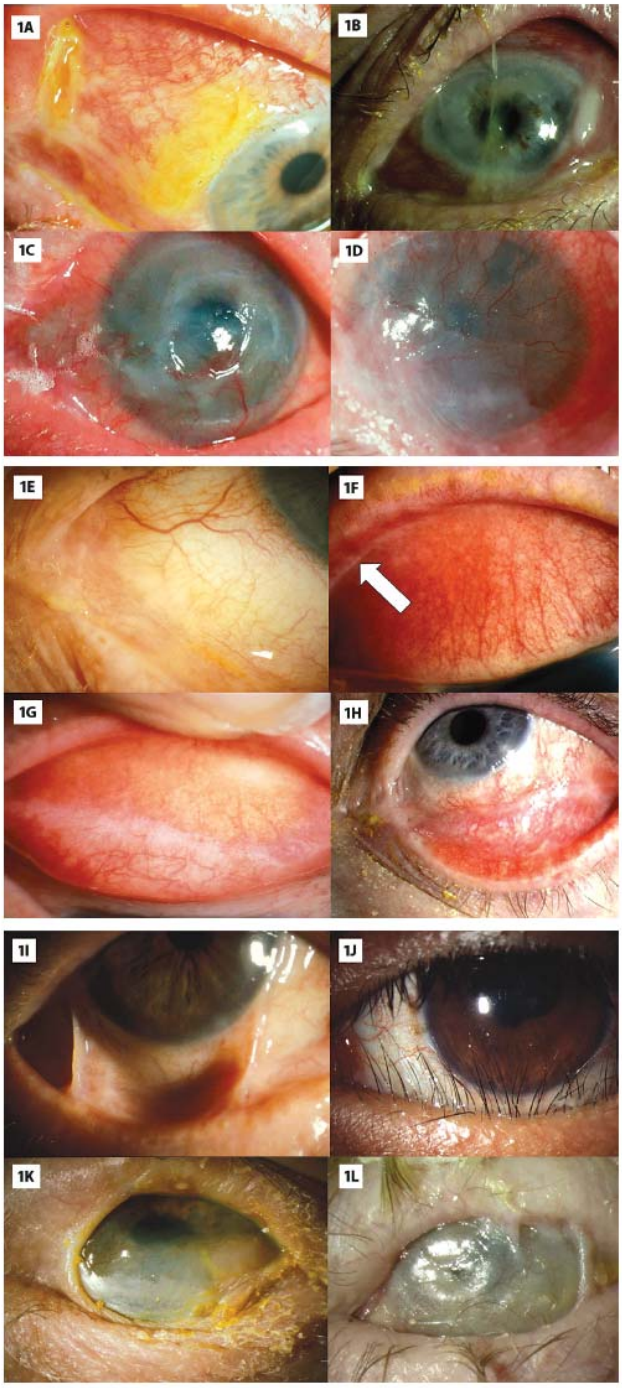
Apart from topical medication-related disease, ocular MMP usually affects both eyes, but the signs and disease progression can be very asymmetric. It is said that the occasional patient presenting with unilateral ocular MMP typically develops fellow eye involvement within 2 years.[14]

B. Immunopathological Investigations

A clinical diagnosis of ocular MMP can be made based on a history of progressive conjunctival scarring and the presence of typical clinical signs after other causes of conjunctival scarring are excluded. Most of the disorders in Table 1 can be identified or excluded by an accurate history, systemic examination, and laboratory investigations. A connective tissue disease antibody screen should be performed, and any skin or mouth lesions should be referred to a specialist for evaluation and biopsy.

1. Histology

Ideally, the clinical diagnosis of MMP is made with laboratory evidence of an autoimmune disease process, with a biopsy from at least one site (skin, buccal, genital, nasopharyngeal



or conjunctival mucosa) being positive on direct immunofluorescence (**DIF**). Positive findings are linear deposition of IgG, IgA, and/or complement along the epithelial basement membrane zone (**BMZ** [Figure 3]). DIF evidence has been recommended as a mandatory requirement for the diagnosis of MMP,[1] as it is almost always positive in MMP that involves tissues other than the eye. However, conjunctival DIF is positive in only 60-80% of cases of ocular MMP,[15,16] and the results can be initially positive then subsequently negative, and vice versa, during the course of the disease, apparently unrelated to disease activity or treatment.[15,17] Thus, ocular MMP cannot be excluded by a negative DIF result.[14]

For these reasons, although a positive DIF result is useful and can distinguish MMP from diseases such as lichen planus, lupus erythematosus or pemphigus vulgaris, which have characteristic immunopathological features of their own,[16] a negative result is not conclusive. However, bulbar conjunctival biopsy is easy and safe, provided the fornix is avoided, and we recommend that both conjunctival[18] and buccal biopsies (occasionally positive in patients without buccal symptoms when conjunctival biopsies are negative[16]) be taken if they can be processed in a laboratory that is experienced in this investigation.

Although DIF for IgG, IgA, and/or complement is characteristic of MMP,[1] identical biopsy findings are found in bullous pemphigoid, linear IgA disease, epidermolysis bullosa, and certain paraneoplastic syndromes,[1] and these diseases must be differentiated by the clinical findings.

Routine histopathology is of little value for the diagnosis of MMP, because the conjunctiva is fragile and detection of basement membrane zone cleavage is unreliable. However, histopathology is mandatory to exclude neoplasia, and it can help diagnose atopic disease and sarcoidosis.

Figure 1. The clinical progression of MMP.

***Acute onset MMP.* A. Ulcerative conjunctivitis. B. Limbitis. C. Rapid progression of conjunctival scarring. D. Surface failure as a sequela of acute uncontrolled limbitis.**

***Subacute and chronic onset MMP.* E. Scarring of the plica in early MMP. F. Scarring in the superior marginal sulcus (indicated by arrow) sometimes occurs early. Conjunctival infiltrate is also present. G. Subconjunctival reticular scarring and conjunctival infiltrate. H. Inferior fornix shortening and conjunctival infiltrate. I. Inferior symblepharon. J. Lower lid entropion and trichiasis. K. Ankyloblepharon. L. End-stage disease with total surface keratinization.**

Table 1. Differential diagnosis of cicatrizing conjunctivitis

1A. Static or very slowly progressive conjunctival scarring
1. Trauma: Physical, chemical, thermal, radiation injury, artefacta
2. Infection: Trachoma, membranous streptococcal and adenoviral conjunctivitis *Corynebacterium diphtheria*, chronic mucocutaneous candidiasis
3. Allergic eye disease: Atopic keratoconjunctivitis
4. Drug-induced conjunctival cicatrization†
5. Mucocutaneous disorders: Stevens-Johnson syndrome and toxic epidermal necrolysis† Graft-versus-host disease
6. Immunobullous disorders: Linear IgA disease,† epidermolysis bullosa acquisita† Dermatitis herpetiformis, bullous pemphigoid Pemphigus vulgaris Discoid and systemic lupus erythematosus*
7. Systemic disease: Rosacea, Sjogren syndrome, inflammatory bowel disease, sarcoidosis, scleroderma, immune complex diseases, ectodermal dysplasia, porphyria cutanea tarda, erythroderma ichthyosiform congenita
1B. Progressive Conjunctival Scarring
1. Neoplasia: Squamous cell carcinoma, sebaceous cell carcinoma, lymphoma
2. Mucous membrane pemphigoid (MMP): a. MMP with ocular involvement b. Ocular MMP associated with other disorders Linear IgA disease Epidermolysis bullosa acquisita Paraneoplastic MMP Drug-induced ocular MMP Stevens-Johnson syndrome
3. Other mucocutaneous & immunobullous disorders: a. Mucocutaneous disorders Lichen planus b. Immunobullous disorders Paraneoplastic pemphigus

† A subset of patients with these diseases may develop autoantibody-positive progressive conjunctival scarring similar to MMP.

* Rare cases can develop progressive scarring.

Figure 2. Unilateral scarring secondary to meibomian gland carcinoma.

2. Serology

Currently, there is no sensitive or specific laboratory test to either establish the diagnosis of MMP or to monitor the response to therapy. This is largely because of the variable clinical and immunological features of MMP, which encompass a spectrum of related phenotypic variants affecting particular mucosal sites and a diversity of target BMZ antigens.[19] Indirect immunofluorescence (**IIF**) to identify circulating autoantibodies in serum can be helpful if positive, but the sensitivity of the technique is poor and partly influenced by whether the substrate used is relevant to the clinical manifestations; even when conjunctiva, the best available substrate for patients with ocular disease, is used, only 41% of samples are positive.[20] Research is under way to develop more sensitive and specific assays for detection of these autoantibodies.[21] If antibody can be detected and a titer obtained, the titers do appear to correlate with disease activity.[22]

III. PRINCIPLES OF MANAGEMENT

Successful management of ocular MMP encompasses:

1) Control of inflammation and fibrosis, the two main pathologic processes leading to progression of scarring to a more advanced stage of disease. Advanced disease is characterized by a dry eye with exposure, surface failure, and corneal blindness, as outlined in Figure 1 and Section IIA. Prompt treatment is required to prevent progression to surface failure, because the prognosis is poor for rehabilitation by corneal graft surgery and/or surface reconstruction.

2) Prompt management and prevention of acute corneal disease secondary to trichiasis and exposure.

3) Facilitation of the physician-patient relationship in what is a lifelong disease.

IV. STRATEGIES FOR MANAGEMENT

Based on the principles stated above, a strategy for management can be identified.

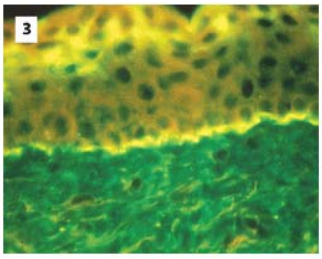


Figure 3. Positive conjunctival direct immunofluorescence with linear deposition of IgG.

A. Control of Factors Leading to Disease Progression

1. Inflammation Associated with Ocular Surface Disease

Untreated inflammation associated with the following conditions impair vision and comfort and also affect the progression of disease and visual loss due to secondary corneal involvement.

a. Blepharitis

Blepharitis is common in MMP and easy to overlook against a background of severe conjunctivitis. Routine treatment methods should be employed.[23,24] The use of oral tetracyclines may improve inflammation related to the underlying immune disorder in MMP.[25] Topical cyclosporin has been shown to improve meibomian gland function and the inflammation associated with blepharitis[24,26] and may be useful in MMP patients.

Blepharitis contributes to colonization with bacterial pathogens, which is one of multiple factors contributing to the constant threat of microbial keratitis. Potential pathogens are recovered from the lids and/or conjunctiva in 85% of OCP patients compared with 49% of controls.[27] Regular bacterial cultures of the eyelids and conjunctiva are an important part of preventive management in OCP. This is particularly crucial prior to any intraocular or periocular surgery.

b. Dry Eye

Dysfunction of several components of the ocular surface-lacrimal gland reflex unit are responsible for dry eye and tear film instability in MMP, contributing to poor quality of vision. Dry eye also predisposes to recurrent epithelial breakdown, to which these eyes with surface failure are already susceptible, and the resultant complications of delayed epithelialization, microbial keratitis, and corneal perforation.

A step-wise approach to management of dry eye underlies the rationale for therapy.[23]

1) *Tear conservation*: Wrap-around glasses and a humidified environment help to conserve tears.

2) *Punctal occlusion*. The punctum is often occluded spontaneously as part of the disease. Occlusion is helpful, provided that blepharitis and its associated conjunctival inflammation have been controlled.

3) Supplementation with tear substitutes. Symptomatic relief of ocular surface symptoms can be provided by tear substitutes. If preserved lubricants are used more than 4-6 times daily, there is a risk of ocular surface toxicity. There are few guidelines for the choice of an optimal unpreserved lubricant for any individual patient. For patients with surface symptoms and minimal aqueous tear deficiency, hypromellose, polyvinyl alcohol, carmellose, carbomer gels, and hyaluronic acid are available without preservatives in some countries. Individual preferences for these among patients vary widely. For patients with severe aqueous tear deficiency, isotonic saline or balanced salt solution (for intraocular use) may be preferred despite their short contact time, as they leave no residue and cause no blurring of vision. Patients with corneal keratinization may prefer ointment for comfort. Soft paraffin may be preferred to the more widely available lanolin and paraffin mixtures because of its reduced viscosity.

4) Topical cyclosporin and steroids to address ocular surface inflammation related to keratoconjunctivitis sicca. Topical cyclosporin (preparation and concentration used according to what is available and tolerated, eg, 0.05% to 2% drops, or 0.2% ointment) or weak topical steroids, such as fluoromethalone or prednisolone 0.5%, can be used for short periods if the inflammation appears to be related to ocular surface dessication.

5) Mucolytics. Debris in the tear film can be cleared by mucolytics, such as acetylcysteine 5% or 10% unpreserved.

6) Lid surgery. Exposure keratopathy due to lagophthalmos and poor Bell's phenomenon needs urgent treatment with lid surgery to prevent corneal ulceration and perforation (Figure 4A and B and Section IV.C).

7) Autologous serum drops. Both subjective and objective improvements in the ocular surface can be achieved in some patients with use of autologous serum drops in 20-100% concentrations.[28] This is probably due to their effect as a physiological, rather than a pharmaceutical tear replacement, as serum contains many of the components of lacrimal tears.

8) Contact lenses. Gas permeable limbal-fit corneal lenses, or scleral lenses,[29,30] may improve surface hydration and vision in some patients. Hydrogel lenses are not recommended in dry eyes, as they lose a percentage of their water content in all eyes and increase tear evaporation. Low water content silicone hydrogels can be used in eyes with at least 5 mm of wetting on a Schirmer I test, when used with frequent application of unpreserved lubricating tears.[31] The increased risk of infection associated with soft lenses compared with rigid lenses must be considered,[32] especially because eyes with MMP are prone to colonization with pathogenic bacteria.[27]

9) Assessment for concomitant Sjogren syndrome. Sjogren syndrome and rheumatoid arthritis can accompany MMP, and they require treatment specific to those conditions.

10) Oral pilocarpine. The parasympathomimetic pilocarpine stimulates tear secretion through the unoccluded lacrimal ductules and thus can be useful for some MMP patients with severe dry eye.[33] The side effects of sweating, flushing, and nausea are minimized when the dose is increased gradually from 5 mg daily for 1 week, then 5 mg bd for 1 week, then 5 mg tid for 1 week, to a maximum dose of 5 mg qid. Patients should also be advised about the potential for more severe side effects, such as tachycardia and bronchospasm.

11) Salivary gland transplantation. Salivary glands have been transplanted into eyes with Schirmer I test measurements of < 2 mm. However, salivary tears do not possess the same nutritional and visual properties as lacrimal tears, and although the eye is wetter, the ocular surface and outcomes of keratoplasty may not be improved.[34] We have abandoned this technique.

c. Filamentary Keratitis and Punctate Epithelial Keratopathy

Filamentary keratitis is largely due to tear deficiency and blepharitis. If it does not improve with treatment of the dry eye and lid margin disease, it often responds to the mucolytic acetylcysteine 5-10% used 1-4 times daily. Therapeutic silicone hydrogel contact lenses can improve the symptoms of filamentary keratitis. With appropriate treatment, this keratitis usually resolves after a few months.

Punctate epithelial keratitis is often secondary to lid margin disease, conjunctival inflammation, topical medication toxicity, and tear deficiency, and it responds to the management of these conditions. Lubricating ointment at night is often helpful.

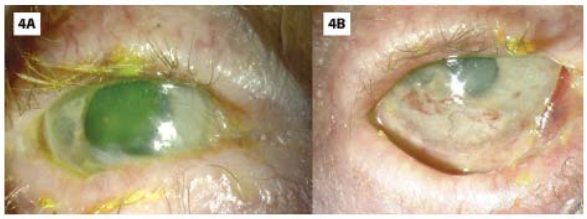


Figure 4. Fornix reconstruction surgery to treat corneal exposure. A. Symblepharon between the cornea and lower eyelid obstructing closure and resulting in lagophthalmos in a patient's only eye. B. Two weeks following buccal mucous membrane graft surgery to reconstruct the lower fornix and amniotic membrane transplantation to the inferior corneal defect. (Reprinted from Saw VPJ, Dart JKG. Management of ocular mucous membrane pemphigoid, Reinhard T, Larkin F (eds). *Cornea and External Eye Disease*. Heidelberg, Springer; 2008, pp 154-175, with the permission of the authors and the publisher.)

d. Keratinization

Keratin occurring on the lid margin or tarsal conjunctiva causes severe discomfort and repetitive trauma to the ocular surface with blinking, and it contributes to corneal scarring and vascularization. Keratinization of the cornea can obstruct vision and cause discomfort. Topical vitamin A as retinoic acid drops 0.05% or 0.01% ointment is effective in about 30% of patients,[35] but an active preparation is not commercially available and very few hospital, or other small manufacturing, pharmacies produce it (Moorfields Pharmaceuticals, 25 Provost Street, London N1 7NG, UK have a preparation, but it is not available for use outside the hospital). It is effective at these concentrations taken once daily or on alternate days, but it may be poorly tolerated. If vitamin A is not available, excessive amounts of keratin can be removed with a blade, but the condition will recur. Scleral or limbal fit contact lenses, gas permeable or soft, can be used to relieve the discomfort.

e. Trichiasis, Entropion, and Lagophthalmos

Untreated trichiasis and entropion lead to chronic ocular surface irritation and a recurrent risk of corneal ulceration and sight-threatening microbial keratitis or perforation. Lagophthalmos (incomplete lid closure) may occur as a result of adhesions between the lid and globe, ie, ankyloblepharon (Figure 1K), or due to scarred and contracted posterior lamellae. Furthermore, impairment of Bell's phenomenon, due to conjunctival shrinkage restricting extraocular motility, also predisposes to corneal exposure. Tear spread and renewal are thus compromised by these eyelid sequelae of MMP, contributing to dry eye-related inflammation. Management of these eyelid sequelae is discussed in detail in section IV.C.

f. Persistent Epithelial Defects and Corneal Perforation

Recurrent or persistent corneal epithelial defects (**PED**s) are responsible for additional ocular surface inflammation and can stimulate the onset of corneal vascularization and surface failure and predispose the cornea to infection and perforation. PED is a major management problem in many patients with severe MMP, and it may be a presenting feature (Figure 5A). Stepwise management, as described below, starts with the simplest therapy and escalates to more complex and invasive treatment, as required.

1) Exclude bacterial or herpes simplex keratitis at the outset. Both may occur, and the clinical signs of keratitis can be suppressed by the use of topical steroids. If such a diagnosis is in doubt, patients should be treated with a short course of intensive broad-spectrum topical antibiotic or oral antiviral agent. Clinicians should have a high index of suspicion for atypical presentations of microbial keratitis in this setting.

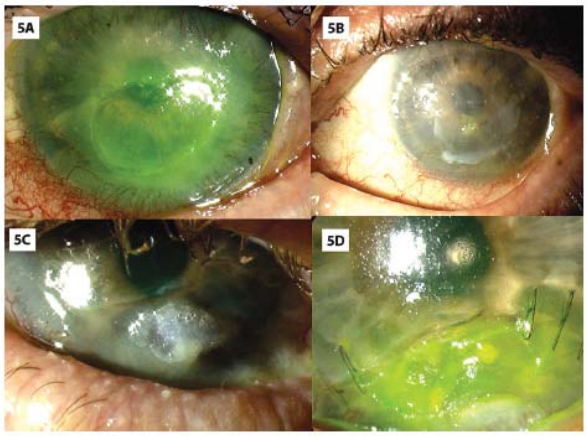


Figure 5. Persistent epithelial defect and management. A. Persistent epithelial defect leading to corneal vascularization and surface failure in the presence of uncontrolled conjunctival inflammation. B. Amniotic membrane graft for persistent epithelial defect. C. Corneal glue for perforation in MMP. D. Lamellar patch corneal graft for perforation in MMP.

2) Correct the precipitating problem: remove ingrowing lashes, prevent corneal exposure (see Section IV.C), treat dry eye.

3) Improve epithelial stability by using frequent non-preserved ointment to reduce shearing forces.

4) Therapeutic contact lens use, appropriate to the degree of dryness of the eye, may be helpful (see Section IV.A.1.b.8).

5) A sutured temporary central tarsorrhaphy can be very effective if contact lenses are inappropriate or fail. Botox protective ptosis is often ineffective, due to fornix shortening.

6) Stimulate epithelialization by controlling inflammation (see Section IV.A.3.a), hence reducing toxic inflammatory mediators.

7) Autologous serum drops, if available, can be effective if previously described measures fail.[28]

8) Improve the basement membrane substrate, over which the epithelium heals. An amniotic membrane graft, within the confines of the persistent defect, combined with a temporary amniotic membrane patch over the whole surface (Figure 5B) or a lamellar corneal graft can be used. A lamellar graft is necessary when there is loss of Bowman's layer due to melt associated with the persistent defect.

9) Failure to heal after the above-described procedures requires renewal of the epithelium by placement of a conjunctival flap or a buccal mucosal graft. Ocular surface reconstruction with corneal stem cells is rarely successful in these circumstances.

10) If corneal perforation occurs, temporize with therapeutic contact lenses or corneal glue (Figure 5C), either of which may be effective. If these measures fail, a patch lamellar tectonic graft (Figure 5D) or penetrating keratoplasty may be necessary. Penetrating keratoplasty should be carried out only if absolutely necessary, because the outcome of tectonic grafts is usually very poor in these eyes, due to failure to epithelialize, melt, infection, vascularization of the graft, and development of glaucoma.[36] If the keratoplasty fails to epithelialize, a conjunctival flap may be needed, but this is usually impossible because of inadequate bulbar conjunctiva. A free buccal mucosal transplant can be performed. Although this is a blinding procedure, it can save an eye for later osteo-odonto keratoprosthesis surgery (see Section IV.D.5).

2. Iatrogenic Toxicity

Treatment toxicity results principally from the preservative benzalkonium chloride (**BAC**), a component of most reusable bottles of eye drop preparations, glaucoma medications, and the aminoglycoside antibiotics.[37] The effects of topical treatment toxicity cannot be distinguished from the effects of the ocular surface disease. Such effects include punctate keratopathy, papillary and follicular conjunctivitis, poor surface wetting, and drug-associated pemphigoid with corneal vascularization. After toxic topical therapy is withdrawn, the mean recovery period is 2 weeks, but may extend to 3 months.[37] Management of toxicity requires avoidance of unnecessary topical treatment and use of unpreserved topical medications, if possible, and alternatives to aminoglycoside antibiotics.

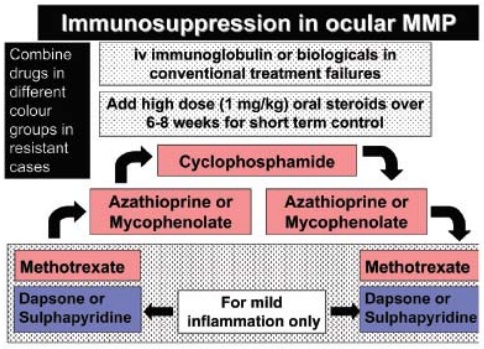


Figure 6. Stepladder immunosuppressive therapy for ocular MMP. For severe disease, commence with cyclophosphamide and plan introduction of less toxic drugs and withdrawal of cyclophosphamide once the disease is under control. For mild disease use dapsone (or sulfapyridine if dapsone is not tolerated) or methotrexate and step-up to azathioprine or mycophenolate. If treatment fails with this, then progress to cyclophosphamide. Oral prednisolone for 6 weeks is usually combined with cyclophosphamide, while awaiting the commencement of immunosuppressive effect. Combinations of a sulpha-based agent (dapsone or sulphapyridine) with a myelosuppressive agent (cyclophosphamide, azathioprine, mycophenolate) and prednisolone are also effective.

3. Immune-Mediated Inflammation

a. Systemic Immunosuppression

Systemic immunosuppression to target inflammation due to the underlying immunological disorder should be instituted after treatment of inflammation related to ocular surface disease. Whether the underlying autoimmune disease is active and requires systemic immunosuppression is best evaluated by examining the upper bulbar conjunctiva, which is least likely to be affected by exposure, trichiasis, and lid margin disease. There is no evidence that topical therapy alters the natural history of the disease.[5] About 25% of MMP patients do not require immunosuppression,[38] as they have mild disease. In end-stage "burned out" disease, immunosuppression is also not necessary, as treatment can only arrest scarring, not reverse it; however, if conjunctival incision surgery, such as fornix reconstruction, is planned in these patients, then immunosuppression must be commenced to prevent a poor surgical result as a consequence of postoperative inflammation and scarring.

We use a stepladder approach to immunosuppressive therapy,[7] depending on the activity of MMP (Figure 6). Briefly, for "*step-up*" therapy, dapsone (diaminodiphenylsulfone, 25-50 mg bid) or sulphapyridine (500 mg od to 500 mg bid, given as sulfasalazine 500 mg bd to 1000 mg bid when sulphapyridine is unavailable) or methotrexate is prescribed for mild or moderate inflammation. If the disease does not respond after 2-3 months of first-line therapy, azathioprine (1-2.5mg/kg/day) is added (when there is some response) or substituted (if there is no response) for these

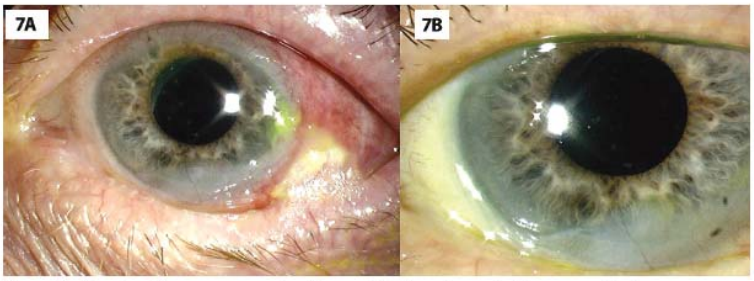


Figure 7. Results of immunosuppressive therapy. A. Inflamed eye prior to commencing immunosuppression. Note the patch of keratinization inferotemporally. B. After 4 months of immunosuppressive therapy, the eye is white and quiet. The superficial corneal vascularization inferiorly, due to partial stem cell deficiency, has not progressed. (Reprinted from Saw VPJ, Dart JKG. Management of ocular mucous membrane pemphigoid, in Reinhard T, Larkin F (eds). *Cornea and External Eye Disease*. Heidelberg, Springer, 2008, pp 154-175, with the permission of the authors and the publisher.)

first-line agents. For patients intolerant of azathioprine, mycophenolate mofetil (500 mg-1 g bd) is effective.[7] Severe inflammatory disease is treated at the top of the stepladder with cyclophosphamide (1-2 mg/kg/day). As optimal effects with cyclophosphamide are not achieved until 8-12 weeks after the initiation of therapy, an adjunctive tapering 6-8-week course of oral corticosteroids (prednisolone commencing at 1-2 mg/kg/day) is employed, sometimes in conjunction with intravenous pulses of methylprednisolone (500 mg-1 g, up to three doses over 3 days).

Because cyclophosphamide is associated with an increased risk of bladder carcinoma, the safe duration of treatment with this agent is limited to 12-18 months. After this, immunosuppression is "*stepped-down*" to the less toxic medications, azathioprine, mycophenolate, methotrexate, or dapsone. Combination therapy is used for resistant cases; this includes the combination of a sulpha (dapsone, sulphapyridine) and myelosuppressive agent (azathioprine, mycophenolate, cyclophosphamide) and/or the addition of prednisolone. High-dose oral corticosteroid does not have a role in long-term management, as adverse side effects preclude sustained use of the high doses required to control disease,[2] and the inflammation quickly recurs when the dose is reduced.

Once complete control of inflammation has been achieved (Figure 7A and B), immunosuppression is continued for at least 12 months. Following this, the dose is slowly tapered and can be stopped if the patient wishes, providing they understand that it must be recommenced if disease activity recurs. Life-long follow-up is necessary, because disease recurs in up to 1/3 of patients.[39] If the disease has been severe and resulted in loss of useful vision in one eye, lifelong therapy is recommended.

Intravenous immunoglobulin appears to be useful for recalcitrant disease[40] or as an alternative to conventional immunosuppression[41]; however, it is too expensive and impractical for use as a routine therapy. In our center, it has been used as adjunctive therapy in patients unresponsive to other drugs with success in 3 of 6 patients (unpublished data). Methotrexate controls inflammation in 89% of mild to moderate disease,[42] but we have concerns about irreversible hepatic and pulmonary fibrosis occurring with prolonged therapy and, therefore, do not use it routinely.

The results of studies reporting the efficacy of each of the different immunosuppressive agents used in MMP are summarized in Table 2. In our recent report of the results of treatment,[7] mycophenolate was used in 34 patients who were intolerant of azathioprine. It was well tolerated and effective, with only 15% of patients developing adverse effects, and a response to treatment was evident in 81% of treatment episodes. In comparison, both dapsone and cyclophosphamide were associated with adverse effects in 31% of our patients (Table 3).

b. Side Effects of Treatment and Monitoring for Toxicity

To screen for drug-related toxic side effects, blood pressure, blood glucose, and blood tests (full blood count with differential, urea and electrolytes, liver function tests) must be evaluated weekly upon the initiation of therapy and biweekly or monthly thereafter in all patients on immunosuppression. Immunosuppressive agents should be administered by physicians experienced in the use of these agents, and many ophthalmologists work with rheumatologists to achieve this. In patients taking cyclophosphamide, lymphopenia is universal and is used to titrate treatment; the target lymphocyte count is 0.5-1.0 x 10^9 cells/mL.[43] The therapeutic goal is isolated lymphopenia, while maintaining a normal total white cell count and neutrophil count. The proportion of patients in our recent study who developed toxicity associated with each of the different immunosuppressive agents is shown in Table 3.

c. Use of Biological Therapies

Monoclonal antibody therapies are potential new treatments for severe refractory MMP. There are four case reports of success using the anti-tumor necrosis factor α agents etanercept[44] and infliximab[45] in patients with severe refractory MMP. Other monoclonal antibody therapies that have been noted in case reports to be effective include rituximab, the anti-CD20 antibody that depletes B cells,[46] and the interleukin-2 antagonist daclizumab.[47]

d. Local Ocular Therapies for Immune-Mediated Inflammation

For mild hyperemia and edema, low-dose topical steroid can be used for comfort if the patient is aware of symp-

tomatic improvement. However, there is no evidence for its efficacy in MMP, the symptoms are likely to recur when treatment is discontinued, and the adverse effects of long-term use (cataract and glaucoma) generally outweigh the benefits. The role of topical cyclosporin in MMP is unclear.[48] A few case reports describe improvement of MMP affecting the eyes and skin following use of topical tacrolimus.[49,50] Theoretically, both cyclosporin and tacrolimus can inhibit local T lymphocyte activation, which has been shown to be present in active MMP.[51]

B. Control of Fibrosis

Currently, the only demonstrated means of slowing the progression of scarring is good control of inflammation with systemic therapy. Without systemic treatment, conjunctival cicatrization progresses in 64% of patients over 10-53 months.[27] With current immunosuppressive regimens, cicatrization can still progress in 11-53% of patients.[7,38,52] Local therapies for conjunctival scarring would be ideal. The use of mitomycin C has been reported,[53-55] but no trials have been carried out, and we have concerns about secondary ischemia, which may affect the success of fornix reconstruction surgery. Other potential therapies require a better understanding of the pathogenesis of the scarring mechanisms.

C. Prophylaxis of Corneal Ulceration and Exposure

Trichiasis can be treated with several methods of lash ablation. For odd lashes or metaplastic lashes, epilation is useful in the short term, but regrowth occurs in 4-6 weeks. Electrolysis or laser thermoablation has a more prolonged effect. Although electrolysis alone has a relatively low success rate of 29%,[56] greater success is achieved when additional cut-down to the lash root is carried out so that electrolysis can be applied to the lash follicle. Argon laser thermoablation has a reported success rate of 50% at 12 months, with further success following retreatment.[57] However, we do not use this treatment routinely. It is important to take care not to cause scarring with excessive use of either electrolysis or laser ablation.

For short lengths of misdirected lashes, lid split through the gray line (the gray line is the lid landmark which demarcates the posterior lamella of tarsus, meibomian gland orifices, and conjunctiva, from the anterior lamella of orbicularis muscle, skin, and eyelashes) and anterior lamellar excision or cryotherapy[58] are effective. We prefer anterior lamellar excision, as it causes less inflammation and less damage to adjacent meibomian glands compared with cryotherapy. Cryotherapy may also cause reactivation of disease. Care should be taken to avoid overtreatment with cryotherapy, as this may cause distortion of eyelid architecture and can contribute to lid margin keratinization.

When there is adequate lid closure and no lagophthalmos, mild-to-moderate cicatricial entropion of the upper lid can usually be repaired by a gray-line lid split and anterior lamellar repositioning, which has a reported success rate of 61%.[59] Lid split and Jones inferior retractor plication for the lower lid has a reported success rate of 54%.[60] Tarsal rotation or excision procedures may sometimes be necessary.[61]

Indications for fornix reconstruction using mucous membrane grafts or other grafts include:

1) the presence of lagophthalmos and a restricted Bell's phenomenon, to prevent exposure keratopathy and corneal perforation (Figure 4A and B).

Table 2. Results of studies reporting the efficacy of immunosuppressive agents used in ocular MMP

Immunosuppressive agent	Study	Disease severity	% (n) of patients responding to treatment
Nicotinamide and tetracycline	Reiche et al[25]	Mild-moderate	50% (n = 8)
Dapsone	Rogers et al[76]	Mild-moderate	83% (n = 24)
	Tauber et al[77]	Mild-moderate	45% (n = 69)
	Foster[5]	Mild-severe	70% (n = 20)
Sulfasalazine	Elder et al[78]	Mild-moderate	50% (n = 20)
	Doan et al[79]	Mild-moderate	45% (n = 9)
Azathioprine	Tauber et al[77]	Mild-moderate	33% (n = 11)
Methotrexate	McCluskey et al[42]	Mild-moderate	88% (n = 17)
Cyclophosphamide and steroids	Elder et al[43]	Severe	15/19 eyes (79%)
	Foster[5]	Severe	100% (n = 12, n = 20)
Tacrolimus	Letko et al[80]	Severe	33% (n = 6)
Cyclosporin	Foster,[5] Foster et al[39]*	Mild-moderate	9% (n =22)
Mycophenolate	Zurdel et al[81]	Severe	9/10 eyes
	Saw et al[7]	Moderate	81% treatment episodes
Intravenous immunoglobulin	Sami et al[40]	Severe	8/8 patients

*Combined results of two case series

2) cicatricial entropion with severe posterior lamellar shortening where anterior lamellar surgery alone would result in lagophthalmos and corneal exposure.

3) when access is necessary for contact lens wear, in preparation for cataract surgery or ocular surface reconstructive surgery with limbal stem cell grafts.

4) to correct ptosis.

Fornix foreshortening per se does not require surgery. This is because of the potential for making the patient worse off rather than better, given that sight-threatening complications can occur.[62] This can be minimized by careful case selection for surgery. Labial or buccal mucous membrane or hard palate or nasal mucosa can be used for posterior lamellar grafting. Nasal mucosal grafts have

Table 3. Proportion of patients developing toxicity with immunosuppressive therapy in ocular MMP[7]

Drug (n = number of patients)	Status [n (%)]	Adverse effect (number of patients with each adverse effect)
Dapsone (n = 90; 2852 patient months)	Discontinued therapy [28 (31%)]	Anemia (8), rash (9), malaise (6), headache (2), diarrhea (2), elevated LFTs (2), thrombocytopenia (1), dysplastic blood film (1), paresthesia (2), back pain (1), stomach cramps (1), worsening of tinnitus (1)
	Continued therapy [5 (5%)]	Anemia (5)
	Total [33 (37%)]	
Sulfapyridine or sulfasalazine (n = 48; 514 patient months)	Discontinued therapy [12 (25%)]	Rash (5), nausea & vomiting (3), malaise (1), depression (1), elevated LFTs & indigestion (1), lymphopenia (1), worsening of tinnitus (1), dizziness (1)
	Continued therapy [3 (6%)]	Nausea (2), anemia (2)
	Total [15 (31%)]	
Cyclosporin (n = 7; 65 patient months)	Discontinued therapy [2 (29%)]	Elevated creatinine (2), hypertension (1)
	Continued therapy [2 (29%)]	Elevated creatinine (2), hypertension (1)
	Total [4 (57%)]	
Methotrexate (n = 4; 58 patient months)	Discontinued therapy [1 (25%)]	Elevated LFTs (1)
	Total [1 (25%)]	
Prednisolone (n = 55; 143 patient months short tapering course, 988 patient months < 20 mg/day low dose)	Discontinued therapy [4 (7%)]	Osteoporosis (2), hallucinations (1), malaise (1)
	Continued therapy [1 (1%)]	Peptic ulcer (1)
	Total [5 (9%)]	
Azathioprine (n = 60; 1230 patient months)	Discontinued therapy [24 (40%)]	Nausea & vomiting (8), headaches (4), elevated LFTs (3), malaise (3), dizziness (2), tinnitus (1), depression (1), diarrhea (1), myelosuppression (1), itch (1), arthralgia (1), myalgia (1), chest & back pain (1), numbness (1), shivering spells (1)
	Continued therapy 2 (3%)]	Dizziness (1), abnormal blood results (1)
	Total [26(43%)]	
Mycophenolate (n = 34; 506 patient months)	Discontinued therapy [5 (15%)]	Malaise (2), diarrhea (1), rash (1), headache (1), shortness of breath and tremor (1)
	Continued therapy [4 (12%)]	Elevated LFTs (1), anemia (1), myelosuppression (1), muscle cramps & insomnia (1)
	Total [9 (26%)]	
Cyclophosphamide (n = 55; 512 patient months)	Discontinued therapy [17 (31%)]	Lymphopenia (5), lethargy & malaise (3), nausea & vomiting (2), diarrhea (1), abdominal discomfort (2), anorexia & weight loss (2), severe pancytopenia (2), anemia (1), elevated LFTs (1), rash (2), headache (1), insomnia (1), myalgia (1), unsteady on feet (1), shortness of breath (1)
	Continued therapy [10 (18%)]	Lymphopenia (5)
	Total [27 (49%)]	

n = number of patients (% of patients receiving drug).
Anemia = hemoglobin < 13.3 g/dL (males) and < 12.0 g/dL (females).
Lymphopenia = < 0.5x 109/L.
LFTs = liver function tests.
No secondary malignancies were detected.

Figure 8. A. Limbal fit rigid gas permeable contact lens protecting cornea against exposure and trichiasis as well as correcting irregular astigmatism. B. Optical penetrating keratoplasty in a wet eye following inferior fornix reconstruction. Note the buccal mucous membrane graft lining the inferior bulbar and tarsal conjunctiva. C. Osteo-odonto keratoprosthesis for endstage MMP.

the reported advantage over buccal mucosa of providing a source of goblet cells with the potential for improving the tear film[63]; however, we do not use nasal mucosal grafts because the mucosa is very thick and the mucus produced is very viscous and does not improve the tear film. Auricular cartilage can also be used as a scaffold for a posterior lamellar mucosal graft when the lid needs to be lengthened.

Careful case selection for mucous membrane grafting is imperative. Heiligenhaus et al[62] reviewed the 2-year results of buccal mucous membrane grafting in 26 eyes. They found that although an improvement was maintained in 35% of eyes (9/26), serious postoperative corneal complications occurred in 62% of eyes (16/26), sometimes requiring permanent tarsorrhaphy. Of the 16 eyes in which fornix reconstruction outcomes were poor, the poor result was associated with the presence of advanced ankyloblepharon (7/16 eyes, 44%) and keratoconjunctivitis sicca (14/16 eyes, 87.5%). When eyes have reached the stage of advanced ankyloblepharon and terminal dryness, fornix surgery is unlikely to have any beneficial effect.

Patients who have a favorable response to immunosuppression are also more likely to show sustained improvement after surgery. Amniotic membrane transplantation, with or without intraoperative mitomycin C, has been used for conjunctival fornix reconstruction in MMP. In our center, the outcomes of amniotic membrane transplantation for fornix reconstruction have not been as promising as those reported by Tseng et al, who have described good results with a long follow-up duration of 16 to 72 months.[64,65]

Lid surgery that does not involve directly operating on the conjunctiva is less likely to reactivate the underlying disease, and it can be performed while the conjunctiva is still inflamed. This includes electrolysis and lid split with anterior lamellar repositioning or inferior retractor plication. Surgery of this nature can be carried out without the need for perioperative steroids or an increase in immunosuppression. However, close postoperative follow-up should be maintained, as inflammation can be induced in some cases, which must be treated aggressively.[59] When incisions of the conjunctiva are necessary, eg, in tarsal rotation surgery and mucous membrane grafting for fornix reconstruction, the disease must be completely controlled by appropriate immunosuppression for at least 2 months prior to surgery, and immunosuppression must be continued during and after the surgery to prevent failure due to uncontrolled inflammation or reactivation of disease. At our center, we use a perioperative 6-week tapering course of oral prednisolone 1 mg/kg/day when conjunctival incision surgery is carried out to prevent or treat an acute reactivation.

D. Improving Vision

Once ocular surface inflammation and progression have been controlled, attention can be turned to other means of improving vision. Moderately advanced conjunctival cicatrization can still be compatible with good vision in MMP. Elder et al found no statistical association between forniceal shortening and visual impairment due to corneal disease.[38] However, when there is extreme loss of the inferior fornix to less than 3 mm, corneal complications are more frequent and severe.

1. Contact Lenses

Rigid gas permeable contact lenses are usually necessary to correct irregular astigmatism associated with corneal scarring in MMP. If fitted to the limbus, these have the additional advantage of protecting against trichiasis and dry eye (Figure 8A). Unfortunately, elderly patients with poor vision may have difficulty wearing contact lenses. If forniceal depth is adequate, scleral gas permeable contact lenses can also correct irregular astigmatism and protect against surface drying, but development of hypoxia and vascularization must be monitored.

2. Cataract Surgery

Cataract is common in MMP. Reasons for this include the elderly patient population, the use of topical and systemic steroids to control inflammation, and the development of cataract following severe keratitis or corneal perforation. Geerling et al[66] and Sainz de la Maza et al[67] have reported the results of cataract surgery in patients with MMP. Visual acuity improved by two or more lines in both series. Conjunctival inflammation should be controlled before surgery, as this can precipitate disease exacerba-

tions. If surgery is limited to corneal incisions only, no additional immunosuppressive therapy is necessary during the perioperative period. If there is active blepharitis, it is recommended that lid cultures be taken 2 weeks prior to surgery and that appropriate topical antibiotic therapy be commenced 7 days prior to surgery. General anesthesia is preferable for cases with short fornices, as 4/0 silk sutures placed through the grey line to retract the lids and canthotomies may be necessary. In these cases, conventional lid specula cannot be retained in the contracted palpebral apertures, and they may cause increased vitreous pressure due to taut symblephara and adhesions.

2% hydroxypropylmethylcellulose spread over the cornea prevents drying during surgery and improves the surgeon's visualization of scarred and irregular corneas. Paraxial rather than coaxial illumination by the operating microscope can dramatically improve the view for phacoemulsification in the presence of irregular astigmatism and opacity.[35] Use of a wick and appropriate head positioning minimizes pooling of fluid, which occurs when silk sutures rather than specula are used to retract the lids. Temporal corneal incisions are used for ease of access when superior and inferior symblepharon are present. Creation of cataract surgical wounds should be avoided in areas of peripheral corneal thinning that may have occurred following keratitis. In such cases, if additional perioperative immunosuppression is given, a scleral tunnel wound can be used. When central corneal scarring is too advanced to permit phacoemulsification, a corneal section extracapsular cataract extraction can be carried out, with a wound enlarged to allow "open sky" surgery, if needed, followed by closure with 10/0 nylon sutures. Trypan blue staining of the anterior capsule assists in visualization of the capsulorrhexis through scarred and irregular corneas.

Extensive canthotomies are sometimes necessary in order to gain access to the globe for surgery. The extent of the canthotomy required is often not realized until surgery is commenced. Large conjunctival defects resulting from canthotomy may be closed with amniotic membrane, combined with topical mitomycin C to the base of the defect. Intracameral antibiotics or intensive topical fluoroquinolone (eg, 1 drop every 2 minutes for 12 minutes) can be used instead of subconjunctival antibiotic injections as prophylaxis against endophthalmitis. Subconjunctival injections are avoided, as these may reactivate conjunctival inflammation. Careful postoperative follow-up is necessary to identify and treat indolent perioperative epithelial defects and any excessive conjunctival inflammation.

3. Corneal Transplant Surgery

Keratoplasty, either penetrating or lamellar, is seldom successful for visual rehabilitation because of the formidable problems of corneal epithelialization in the poor ocular environment that accompanies MMP. Dry eyes have a very poor prognosis for success following keratoplasty. Failure to epithelialize predisposes to corneal vascularization, melt, desmetocele formation, and perforation. Microbial infection is frequent. Invasion of the corneal surface by vascularized conjunctival epithelium, due to corneal epithelial stem cell deficiency, also results in vascularization and opacification of the corneal graft.

Tugal-Tukan et al reported results of keratoplasty in nine eyes with MMP.[68] Despite aggressive preoperative treatment, including systemic immunosuppression, mucous membrane grafts, and lash cryotherapy, there was a high rate of complications with generally poor visual results. Five eyes developed PEDs, leading to ulceration in four cases. One required a keratoprosthesis and five required a further tectonic regraft. Vision was improved in three eyes, unchanged in three, and worse in three eyes. The results of tectonic penetrating keratoplasty in six eyes have been reported by Macleod et al.[36] Although three patients gained visual improvement from the procedure, this was not maintained, due to progressive vascularization of the graft, melt leading to desmetocele formation, and development of glaucoma.

Careful case selection for keratoplasty is critical. In our center, we have recently carried out three penetrating keratoplasties for visual rehabilitation in eyes with MMP and adequate tear production indicated by a Schirmer I test $\geq$ 5 mm. In all three eyes, fornix reconstruction surgery was performed preoperatively to ensure good lid closure, and the keratoplasty surgery was carried out under increased systemic immunosuppression. With a follow-up of 2-3 years, all three grafts have improved vision by 2 to 6 lines.

4. Ocular Surface Reconstructive Surgery

Chronic inflammatory insult to the limbal epithelial stem cells is a cause of surface failure and an important reason for failure of keratoplasty surgery in eyes with MMP. Improved outcomes have been reported with reconstruction of the ocular surface by removal of the fibrovascular pannus that obstructs vision, transplantation of limbal stem cells, and lamellar or penetrating keratoplasty for stromal corneal vascularization and scarring. Tsubota et al reported successful visual improvement in 9/9 eyes with MMP with use of limbal allografts and amniotic membrane transplantation in conjunction with systemic immunosuppression with dapsone and cyclosporin.[69] Penetrating keratoplasty was also performed in 5 of the 9 eyes. However, these promising results at 5 months were not sustained with prolonged follow-up. The same authors reported that surface reconstruction using this technique was successful in 41% of a group that included both Stevens-Johnson syndrome and MMP patients, at an average follow-up of 3 years.[70] Other reports of the outcomes of surface reconstructive surgery that used living-related limbal allografts[71] or cultivated epithelial stem cells[72,73] describe results for only two or three MMP eyes, and it is not possible to make conclusions from these reports. When this surgery fails, placement of a buccal mucosal flap is usually required to prevent corneal perforation. Osteo-odonto keratoprosthesis (**OOKP**) surgery can be used after a buccal mucosal flap (see below).

5. Keratoprosthesis

For dry eyes with MMP, keratoprosthesis surgery can restore vision. Usually, by this stage, the disease has burned out. If not, it is important that any disease activity is controlled with adequate immunosuppression before surgery is performed. A satisfactory keratoprosthesis for patients with MMP is the OOKP, originally described by Strampelli. It is a heterotopic autograft, in which the cornea is replaced by a polymethylmethacrylate (**PMMA**) optical cylinder glued to a biological support (haptic) made of human living tissue (autologous osteodental lamina). Recent long-term results reported by Falcinelli et al,[74] including 39 eyes with MMP, indicate an 85% probability of retaining the OOKP 18 years after surgery, with a mean best corrected visual acuity of 0.8 at 10 years in MMP patients.

Compared with other keratoprostheses, OOKP has a low risk of infection and extrusion, and it is particularly resilient to the hostile environment of a dry keratinized eye. Its main disadvantages are that the surgery involved is high-risk and extensive, cosmesis is poor, and the field of vision is limited. Successful long-term visual outcome in MMP patients using the Dohlman keratoprosthesis has also been reported[75]; however, complications are not uncommon. These include glaucoma, necrosis of surrounding tissue, aqueous leak and endophthalmitis, retinal detachment, and prosthesis extrusion.

V. CONCLUSION

Both the diagnosis and management of ocular MMP present challenges to the anterior segment clinician. There is little to offer patients with advanced disease who have progressed to the stage of a terminally dry eye, stem cell failure, and ankyloblepharon. For these reasons, the key to management is early diagnosis and control of inflammation and scarring. Current research into the autoantibody response to epithelial basement membrane epitopes is likely to lead to improvements, both in diagnosis and in our understanding of disease pathogenesis. Although immunosuppressive therapy is successful in controlling inflammation in a large proportion of patients, it takes several weeks to become effective, during which time the disease can progress rapidly and lead to blinding sequelae. Biologic therapies show promise in recalcitrant disease, but large international studies may be necessary to show good evidence of effect. Moreover, even with clinical control of inflammation, fibrosis can still progress, leading to the development of advanced disease; the reasons for this are uncertain and improved understanding of the fibrotic process is needed to further the development of antifibrotic therapies.

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Immunosuppressive Therapy for Ocular Mucous Membrane Pemphigoid

Strategies and Outcomes

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Purpose: To evaluate the effectiveness and toxicity of a stepladder immunosuppression strategy, including the use of mycophenolate mofetil and combination therapy, in the treatment of ocular mucous membrane pemphigoid.

Design: Retrospective, noncomparative, interventional case series.

Participants: Two hundred twenty-three eyes of 115 patients.

Methods: Patients with a diagnosis of ocular mucous membrane pemphigoid commencing immunosuppression between January 1994 and July 2005 were identified. A treatment episode was defined by the use of a particular therapy or combination of therapies.

Main Outcome Measures: For each treatment episode, success of immunosuppressive therapy in controlling ocular inflammation was graded as a success (S), qualified success (QS), or failure (F). Initial and final visual acuities (VAs), stage of cicatrization (Foster, Mondino), grade of conjunctival inflammation, and side effects were recorded.

Results: In 70% (80/115) of patients, inflammation was controlled by the end of the study. At least 6 months remission off treatment occurred in 16 patients (14%). Of the 388 treatment episodes, 50% were classified as S; 27%, QS; and 23%, F. The most successful therapies were based on cyclophosphamide (S, 69%; QS, 21%; F, 10%), followed by mycophenolate (S, 59%; QS, 22%; F, 19%), azathioprine (S, 47%; QS, 24%; F, 29%), dapsone (S, 47%; QS, 30%; F, 23%), and sulfapyridine (S, 38%; QS, 27%; F, 35%). Combination sulfa–steroid–myelosuppressive agent therapy increased the response from 73% with single-agent therapy to 87%. Side effects were the reason for 29% of changes in therapy. These were most prominent with azathioprine (40%) and least with mycophenolate (15%). Initial best-corrected VA (BCVA) was 6/60 or less in 17% (37/223) of eyes, pemphigoid being the cause in 13% (29/223). Final BCVA was 6/60 or less in 34% (76/223) of eyes, pemphigoid being the cause in 26% (57/223). By the end of the study, Mondino stage cicatrization had progressed in 41% (92/223) of eyes and 53% (61/115) of patients.

Conclusions: Mycophenolate mofetil seems to be an effective and well-tolerated immunosuppressant for moderately active ocular mucous membrane pemphigoid. Combination sulfa–steroid–myelosuppressive agent therapy in a stepladder regimen is a useful strategy to improve disease control. Cicatrization and VA may still progress and worsen despite adequate control of inflammation. *Ophthalmology 2008;115:253–261 © 2008 by the American Academy of Ophthalmology.*

Ocular mucous membrane pemphigoid (MMP), also known as ocular cicatricial pemphigoid (OCP), is part of a systemic cicatrizing autoimmune disease involving the mouth, skin, and other mucous membranes. The natural history of disease involving the eye is chronic progressive cicatrization,

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leading to blindness caused by surface failure, corneal vascularization, and opacification.[1,2] The main goals of therapy are suppression of inflammation and prevention of cicatrization. Episodes of acute inflammation punctuating the chronic progressive course are associated with rapid conjunctival scarring.[3] Conjunctival cicatrization can, however, progress despite immunosuppressive treatment.[4,5]

Inflammation resulting from systemic immunodysregulation requires treatment with immunosuppressive agents, after addressing inflammation caused by surface disease (blepharitis, trichiasis, dry eye), infection, and toxicity. Almost 75% of patients require systemic immunosuppression,[6] and 46% require continuing systemic treatment to avoid disease reactivation.[7] Evidence for the use of current immunosuppressive and immunomodulatory therapy in OCP comes from cohort studies,[8,9] interventional case series and nonrandomized studies,[4,5,10–13] retrospective case series,[14,15] and 2 randomized trials.[1] These have indicated a role for dapsone, sulfasalazine or sulfapyridine, or methotrexate in mild to moderate disease; azathioprine in moderate disease; cyclophosphamide with a short course of prednisolone for severely active disease; and intravenous immunoglobulin for refractory disease.

Mycophenolate mofetil recently was reported to be effective in OCP in small case series that included results in 10 patients with ocular involvement.[16–18] How mycophenolate compares, in terms of efficacy and frequency of adverse effects, with dapsone, azathioprine, and other agents used to control inflammation in OCP is unknown. Combination therapy using more than one immunosuppressive agent may be necessary for control of inflammation.[7,15] It is not known which combinations may be most effective and which are associated with the least toxicity.

The purpose of this study was to evaluate, by retrospective review, the effectiveness and toxicity of current immunosuppressive regimens, including use of mycophenolate mofetil and combination therapy, employed to control inflammation in OCP. From this, the authors make recommendations regarding the value of a step-up and step-down strategy to guide clinicians using immunosuppressive therapy in OCP.

Patients and Methods

The clinical records of 115 consecutive patients with ocular MMP attending Moorfields Eye Hospital from January 1994 through August 2005 were reviewed retrospectively. Inclusion criteria were patients with a clinical diagnosis of ocular MMP and at least 6 months of follow-up after commencing immunosuppression.

A clinical diagnosis of ocular MMP was made if there was progressive conjunctival cicatrization, after excluding other causes of progressive scarring[19] such as Sjögren's syndrome, atopic keratoconjunctivitis, and rosacea. Drug-induced ocular MMP was diagnosed in patients with clinical features of OCP who had a history of causative medication use and progression of clinical signs despite cessation of the causative therapy.

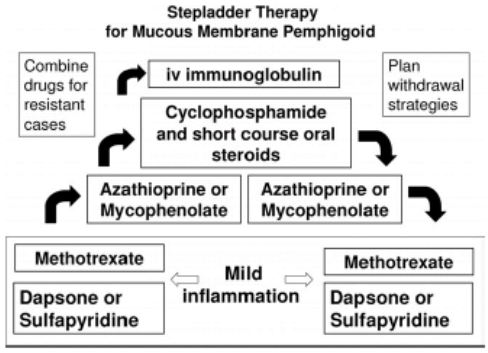

Figure 1. Algorithm showing stepladder immunosuppression strategy. For severe disease, commence with cyclophosphamide and plan introduction of less toxic drugs and withdrawal of cyclophosphamide after the disease is under control. For mild disease, use dapsone (or sulfapyridine if dapsone is not tolerated) and step up to azathioprine or mycophenolate. For treatment failures with this regimen, progress to cyclophosphamide. Oral prednisolone for 6 weeks is usually combined with cyclophosphamide while awaiting the commencement of immunosuppressive effect. Combinations of a sulfa-based agent (dapsone or sulfapyridine) with a myelosuppressive agent (cyclophosphamide, azathioprine, mycophenolate) and prednisolone also are effective. iv = intravenous.

All reasonable attempts were made to obtain a positive immunopathologic tissue diagnosis[20]; however, negative or inconclusive biopsy results did not exclude the diagnosis of OCP[21,22] in the presence of characteristic clinical features.

Data collected included date of diagnosis, biopsy results, extraocular manifestations, details of therapies, treatment-related side effects, and reasons for changes in therapy. The ocular findings analyzed included best-corrected visual acuity, conjunctival inflammation, and the most advanced Mondino and Foster stage; all were noted at the first visit and at last follow-up. Conjunctival inflammation was graded as 0 (absent) through 4 (severe). The worse eye of each patient was selected according to the following criteria: higher Mondino stage, worse inflammation, worse visual acuity, or the right eye if both eyes were equal.

Immunosuppressive regimens used either step-up or step-down treatment depending on the activity of OCP, as previously described (Fig 1).[23] Briefly, for step-up therapy, dapsone (25–50 mg twice daily) or sulfapyridine (500 mg once to twice daily, or sulfasalazine 500–1000 mg twice daily) was prescribed for mild or moderate inflammation. For disease not responding after 2 to 3 months of first-line therapy, azathioprine (1–2.5 mg/kg daily) was added when there was some response or was substituted if there was no response. For those intolerant of or not responding to azathioprine, mycophenolate mofetil (500 mg–1 g twice daily) was used. Severe inflammatory disease was treated with cyclophosphamide (1–2 mg/kg daily). Because optimal effects with cyclophosphamide are not achieved until 6 weeks after the initiation of therapy, an adjunctive reducing regi-

men of oral corticosteroids (prednisolone commencing at 1 mg/kg daily) was used, sometimes with intravenous pulses of methylprednisolone (500 mg–1 g, up to 3 doses over 3 days). Because of an increased risk of bladder carcinoma,[24] the safe duration of treatment with cyclophosphamide is limited to 12 months, so immunosuppression was stepped down to the less toxic medications azathioprine, mycophenolate mofetil, methotrexate, or dapsone at the end of this period. Combination therapy was used for resistant cases and included the combination of a sulfa (dapsone, sulfapyridine) and myelosuppressive agent (azathioprine, mycophenolate, cyclophosphamide), the addition of prednisolone (either as a maintenance dose of ≤7.5 mg/day or as a brief tapering 6–8-week course), or both. Blood pressure, weight, urinalysis, and blood test results were evaluated regularly to screen for drug-related side effects.

A treatment episode was defined by the use of a single particular immunosuppressive agent or therapeutic combination of agents. For each treatment episode, the success of immunosuppressive therapy in controlling inflammation was classified as a success (S), qualified success (QS), or failure (F). Success (S) was defined as an induction of quiescence with a quiet, white eye for at least 3 months from the commencement of therapy. If, after initial S, a change in therapy was necessary because of a reactivation of inflammation uncontrolled by the current regimen, this was defined as S then inflammation (S_i); if a change in therapy, after initial S, was necessary because of adverse effects, this was defined as S then adverse effects (S_a). Qualified success (QS) was defined as inflammation that was partially controlled, with some residual inflammation. Failure was defined as either no response and persistent inflammation (F_i) or the withdrawal of therapy within 3 months because of unacceptable side effects before a response could be expected (F_a).

Treatment episodes were defined according to the principal agent used (cyclophosphamide, azathioprine, mycophenolate, sulfapyridine or sulfasalazine, or dapsone), either as monotherapy or in combination therapy. When treatment was stopped after a period of quiescence, and if it was not necessary to reinstitute immunosuppression for at least 6 months, disease was described as being in remission. The induction of quiescence was a criterion for S, rather than the permanence of quiescence.

The study was conducted after approval from the local research ethics committee. The Fisher exact test was used to assess for any association between outcome after the first treatment episode and treatment. The Wilcoxon rank-sum test was used to assess for any evidence of association between progression of cicatrization (defined by progression in Mondino stage) and each of maximum recorded level of inflammation and duration of disease. All analyses were conducting using Intercooled Stata software version 9.0 for Windows (StataCorp LP, Texas, LA).

Results

One hundred fifteen patients received immunosuppression. The female-to-male ratio was 1.25:1. The mean age at diagnosis was 66.9 years (standard deviation, 14.4 years; range, 17–92 years). Unilateral disease was present in 6% (7/115) of patients, of whom 1 had unilateral drug-induced pemphigoid. There were 3 patients with drug-induced pemphigoid and 2 patients with OCP developing as a sequela of Stevens-Johnson syndrome. The median duration of disease was 4.25 years, with an interquartile range of 2.17 to 8.67 years. For 90% of patients, the duration of disease was within 14 years, and the longest duration was 25 years (1 patient).

Diagnosis

Of the 62 patients for whom biopsy results were available, 69% (43/62) were positive (see Excel table [available at http://aaojournal.org]). Two patients had positive histologic results. Direct immunofluorescence (DIF) results in the remaining 41 patients were positive. Conjunctival biopsies had been performed in 79% (49/62) of these patients, of which 61% (30/49) showed positive DIF results. Of the 14 buccal biopsy results available, 78.6% (8/14) were positive on DIF microscopy, whereas 57.1% (4/7) of the skin biopsy results were positive using DIF.

Extraocular Involvement

Extraocular manifestations were present in 50% (58/115) of patients. These included lesions of the mouth in 78%, the pharynx in 15%, the esophagus in 14%, the nose in 33%, the skin in 22%, the larynx in 14%, the rectum in 5%, and the genitals in 9%. Associated autoimmune disease was present in 16% (18/115) of patients.

Treatment

Three hundred eighty-eight treatment episodes occurred. Details of immunosuppressive therapies given to each patient are shown in an Excel table (available at http://aaojournal.org). Twelve individual agents were used in 45 different therapeutic permutations, dictated by both disease severity and medication toxicity. A successful outcome occurred in 50% (196/388) of treatment episodes, and 27% (104/388) were considered a QS. Inflammation thus responded to immunosuppression in 77% (300/388) of the treatment episodes. Treatment failed in 23% (88/388) of episodes, of which 51% (45/88) failed because of adverse side effects (F_a), and 49% (43/87) failed because of inability to control inflammation (F_i). The predominant outcome of each patient's treatment course, calculated by determining the outcome present for the greatest duration of the treatment course, was S in 75% (86/115), QS in 20% (23/115), and F in 5% (6/115).

In all patients, for the first treatment episode, use of cyclophosphamide (S, 44%; QS, 37%; F, 19%) and azathioprine (S, 42%; QS, 25%; F, 33%) in severe and moderate inflammation, respectively, seemed to be more successful than use of dapsone (S, 33%; QS, 33%; F, 34%) or sulfapyridine (S, 30%; QS, 27%; F, 43%) in cases of mild inflammation (Fig 2). There was no statistical difference in outcomes according to treatment given ($P = 0.842$, Fisher

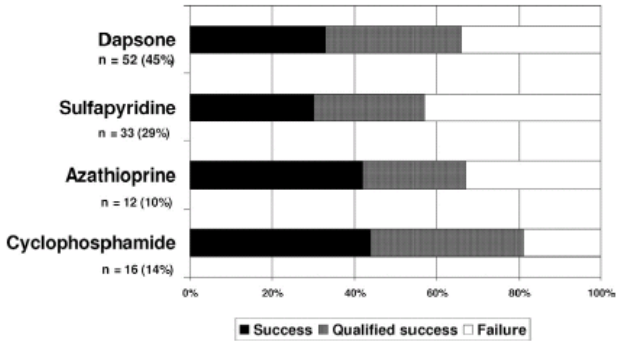

Figure 2. Bar graph showing first treatment episode outcomes for all patients. Cyclophosphamide and azathioprine seem to be more successful than dapsone and sulfapyridine, but there was no significant association between outcomes and treatment given ($P = 0.842$). x-axis, percentage success, qualified success, or failure in patients given that agent; y-axis, principal agent.

exact test). First treatment episode outcomes for the biopsy-positive patients alone are shown in Figure 3 (available at http://aaojournal.org). In the biopsy-positive patients, sulfapyridine (S, 40%; QS, 30%; F, 30%) seemed to be more successful than dapsone (S, 33%; QS, 33%; F, 33%), whereas azathioprine (S, 33%; QS, 50%; F, 17%) and cyclophosphamide (S, 33%; QS, 50%; F, 17%) showed similar results. The proportion of patients receiving each of the different principal agents was similar in both the biopsy-positive group and the overall group; for example, cyclophosphamide was used as a first-line agent in 14% of patients in both groups.

The overall S, QS, or F of treatment in controlling inflammation, based on the principal agent used, for all patients is shown in Figure 4. Although used in the most severely inflamed cases, cyclophosphamide (S, 69%; QS, 21%; F, 10%) seemed to be the most successful, followed by mycophenolate (S, 59%; QS, 22%; F, 19%), azathioprine (S, 47%; QS, 24%; F, 29%), dapsone (S, 47%; QS, 30%; F, 23%), and sulfapyridine (S, 38%; QS, 27%; F, 35%). Similar analysis for the biopsy-positive patients alone is shown in Figure 5 (available at http://aaojournal.org). Again, both cyclophosphamide (S, 77%; QS, 18%; F, 5%) and mycophenolate (S, 73%; QS, 18%; F, 9%) seemed to be very successful, whereas the results for azathioprine (S, 48%; QS, 22%; F, 30%), dapsone (S, 43%; QS, 36%; F, 21%), and sulfapyridine (S, 39%; QS, 28%; F, 33%) were comparable.

There was no evidence from the data of any association between outcome and gender or between outcome and age at commencement of treatment, although the study was not specifically designed to detect this. Mondino and Brown[2,5] reported that disease at more advanced stages of cicatrization has a more rapidly progressive course, so the efficacy of therapy may be evaluated best when comparing outcomes in eyes at similar stages of cicatrization. For each principal agent, there was no evidence from the data of any significant differences in outcomes according to the stage of cicatrization, although the study was not designed to detect this.

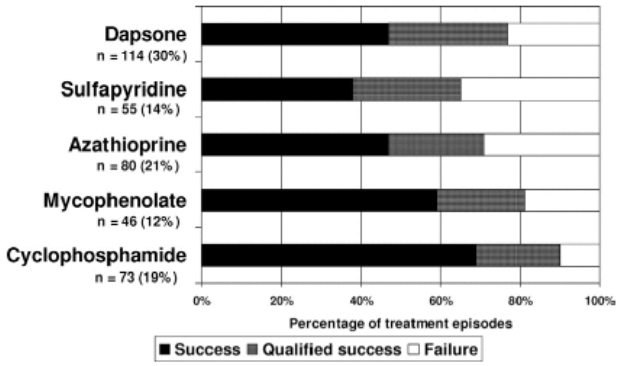

Figure 4. Bar graph showing overall outcomes for all patients, according to principal agent given. The study was not designed to compare overall outcomes of treatments, so statistical analysis was not appropriate. n = no. of treatment episodes (% of all treatment episodes).

Table 1. Reasons for Further Therapy

Reason for Further Therapy	n (%)*
Failure because of poor inflammatory control (F_i)	40 (14.7%)
Failure because of adverse side effects (F_a)	42 (15.4%)
Incomplete response (QS)	90 (33.0%)
Reactivation after initial success (S_i)	25 (9.2%)
Adverse side effects after initial success (S_a)	38 (13.9%)
Maximum duration cyclophosphamide therapy	21 (7.7%)
Remission then relapse	5 (1.8%)
Patient stopped therapy then required recommencement	3 (1.0%)
Physician choice or other reason	9 (3.3%)

*Number of treatment episodes (% of treatment episodes requiring further therapy).

Stepladder Therapy

Of the 115 patients who received treatment, 26 patients (23%) underwent only 1 treatment episode, 30 patients underwent 2 treatment episodes (26%), and the number of treatment episodes increased to a maximum of 10 episodes in 3 patients (3%; see Excel table [available at http://aaojournal.org]). The reasons for further therapy are shown in Table 1. After the first treatment episode, immunosuppressive treatment was stepped up in 52% (60/115) of patients, stepped down in 10% (12/115) of patients, and remained at the same level on the therapeutic stepladder in 38% (43/115) of patients.

Single-Agent and Combination Therapy

Single agents were used in 53% (204/388) of treatment episodes, approximately as commonly as combined agents. The outcomes of particular combinations of therapy compared with single-agent therapy, for all treatment episodes, are shown in Figure 6 (available at http://aaojournal.org).

Final Outcome

The final outcome of treatment was a white quiet eye in 70% (81/115) of patients and 78.5% (175/223) of eyes. There was residual minimal or mild inflammation in 21.5% (48/223) of eyes and 30% (34/115) of patients. Of the 48 eyes with residual inflammation, 26 (54%) continued to receive treatment, treatment ceased in 16 (33%) eyes, and 6 eyes (3 patients) were lost to follow-up. In the 16 residually inflamed eyes in which treatment had ceased, side effects prevented further treatment in 5 eyes (3 patients), other illness unrelated to therapy resulted in treatment cessation in 2 eyes (1 patient), the residual inflammation was minimal and fluctuating and the risks of treatment were believed to outweigh the benefits in 6 eyes (4 patients), and the patient wished to stop treatment in 3 eyes (3 patients). By the end of the study, 43.5% (50/115) of patients had ceased treatment. Of these 50 patients, both eyes were white in 70% (35/50) and 1 or both eyes were residually inflamed in 30% (15/50). Of the 65 patients (56.5%) in whom treatment was continuing, both eyes were white in 71% (46/65), and 1 or both eyes were residually inflamed in 29% (19/65). Remission off treatment for at least 6 months occurred in 16 patients (14%). Four additional patients experienced relapses after periods of remission off treatment lasting from 4 to 24 months. Thus, the proportion in remission was 17% [(16+4)/115], and relapse occurred in 20% (4/20).

Side Effects

The adverse effects of therapy are listed in Table 2. Three patients experienced major adverse effects. Two patients receiving cyclophosphamide experienced pancytopenia requiring transfusion. One of these patients did not attend several clinic appointments while continuing to receive cyclophosphamide. The other patient experienced pancytopenia and hepatotoxicity. A third patient experienced severe anemia during sulfapyridine treatment; therapy was later changed to cyclophosphamide for pemphigoid-related airway obstruction, of which she died. No patients developed secondary malignancies.

Cicatrization

Conjunctival cicatrization at presentation was Mondino stage I in 13%, stage II in 43%, stage III in 28%, and stage IV in 16% of eyes, and Foster stages I through IV in 2%, 15%, 81%, and 2% of eyes, respectively. Progression of Mondino stage occurred in 53% of patients (61/115) and 41% of eyes (92/223), whereas Foster stage progressed in 8% of patients (9/115) and 4% of eyes (9/223). Using Mondino's staging system, progression occurred in 63% (19/30) of stage I eyes, 48% (45/93) of stage II eyes, 43% (25/58) of stage III eyes, and none of the stage IV eyes. Using Foster's staging system, progression occurred in 20% (1/5) of stage I eyes, 18% (6/33) of stage II eyes, 1% (2/180) of stage III eyes, and none of the stage IV eyes. We found evidence that progression of cicatrization in the worse eye was associated with less severe inflammation ($P = 0.0007$) and longer duration of disease ($P = 0.0048$; Table 3).

Visual Acuity

The best-corrected visual acuity at presentation was better than 6/18 in 67% of eyes, 6/18 to 6/60 in 16% of eyes, 3/60 to 6/60 in 5% of eyes, and less than 3/60 in 11% of eyes. The final best-corrected visual acuity was better than 6/18 in 48% of eyes, 6/18 to 6/60 in 19% of eyes, 3/60 to 6/60 in 7% of eyes, and less than 3/60 in 26% of eyes. Ocular MMP was the cause of blindness (acuity, less than 3/60) in 8% of eyes at presentation and in 21% of eyes at the final visit. During the course of follow-up, the proportion of eyes with visual acuity 6/60 or less because of OCP increased from 13% (29/223) to 26% (57/223), whereas the proportion of eyes with visual acuity of 6/60 or less from other causes (retinal vascular event, glaucoma, macular degeneration) increased from 4% (8/223) at presentation to 7% (16/223) at the final visit. The best recorded acuity often required contact lens correction, but not all patients could tolerate contact lenses, so a greater proportion of eyes had worse functional visual acuities.

Table 2. Adverse Side Effects

Drug	Status	n (%)	Adverse Effects
Dapsone (n = 90; 2852 patient months)	Discontinued	28(31%)	Anemia (n = 8), rash (n = 9), malaise (n = 6), headache (n = 2), diarrhea (n = 2), elevated LFTs (n = 2), thrombocytopenia (n = 1), dysplastic blood film (n = 1), paraesthesia (n = 2), back pain (n = 1), stomach cramps (n = 1), worsening of tinnitus (n = 1)
	Continued	5(5%)	Anemia (n = 5)
	Total	33(37%)	
Sulfapyridine or sulfasalazine (n = 48; 514 patient months)	Discontinued	12(25%)	Rash (n = 5), nausea & vomiting (n = 3), malaise (n = 1), depression (n = 1), elevated LFTs & indigestion (n = 1), lymphopenia (n = 1), worsening of tinnitus (n = 1), dizziness (n = 1)
	Continued	3(6%)	Nausea (n = 2), anemia (n = 2)
	Total	15(31%)	
Ciclosporin (n = 7; 65 patient months)	Discontinued	2(29%)	Elevated creatinine (n = 2), hypertension (n = 1)
	Continued	2(29%)	Elevated creatinine (n = 2), hypertension (n = 1)
	Total	4(57%)	
Methotrexate (n = 4; 58 patient months)	Discontinued	1(25%)	Elevated LFTs (n = 1)
Prednisolone (n = 55; 143 patient months short tapering course, 988 patient months <20 mg/day low dose)	Discontinued	4(7%)	Osteoporosis (n = 2), hallucinations (n = 1), malaise (n = 1)
	Continued	1(1%)	Peptic ulcer (n = 1)
	Total	5(9%)	
Azathioprine (n = 60; 1230 patient months)	Discontinued	24(40%)	Nausea & vomiting (n = 8), headaches (n = 4), elevated LFTs (n = 3), malaise (n = 3), dizziness (n = 2), tinnitus (n = 1), depression (n = 1), diarrhea (n = 1), myelosuppression (n = 1), itch (n = 1), arthralgia (n = 1), myalgia (n = 1), chest & back pain (n = 1), numbness (n = 1), shivering spells (n = 1)
	Continued	2(3%)	Dizziness (n = 1), abnormal blood results (n = 1)
	Total	26(43%)	
Mycophenolate (n = 34; 506 patient months)	Discontinued	5(15%)	Malaise (n = 2), diarrhea (n = 1), rash (n = 1), headache (n = 1), shortness of breath & tremor (n = 1)
	Continued	4(12%)	Elevated LFTs (n = 1), anemia (n = 1), myelosuppression (n = 1), muscle cramps & insomnia (n = 1)
	Total	9(26%)	
Cyclophosphamide (n = 55; 512 patient months)	Discontinued	17(31%)	Lymphopenia (n = 5), lethargy & malaise (n = 3), nausea & vomiting (n = 2), diarrhea (n = 1), abdominal discomfort (n = 2), anorexia & weight loss (n = 2), severe pancytopenia (n = 2), anemia (n = 1), elevated LFTs (n = 1), rash (n = 2), headache (n = 1), insomnia (n = 1), myalgia (n = 1), unsteady on feet (n = 1), shortness of breath (n = 1), dysuria (n = 1)
	Continued	10(18%)	Lymphopenia (n = 5)
	Total	27(49%)	

LFTs = liver function tests.
Anemia, hemoglobin <13.3 g/dl (males) and <12.0 g/dl (females). Lymphopenia, $<0.5\times10^9$/l.

Discussion

Stepladder Therapy

Use of the step-up and step-down strategy was completely or partially successful in controlling inflammation in 95% (109/115) of patients in this study. Seventy-seven percent (300/388) of treatment episodes were successes or partial successes. Despite this, progression of cicatrization was evident in 41% (92/223) of eyes and 51% (61/115) of patients. The proportion of patients experiencing adverse effects was 29%, and most of these resolved on cessation of therapy.

Results for Individual Drugs

The results for dapsone, sulfapyridine, and azathioprine treatment are in agreement with those of previous reports.[1,10,11] Mycophenolate was used in this study as an alternative in patients intolerant of or not responding to azathioprine. One of our most important findings is that mycophenolate appears to be a very effective agent for control of inflammation in ocular MMP. Mycophenolate also was associated with the lowest frequency of side effects (15%). To our knowledge, this is the largest reported series of MMP patients (n = 34) treated with mycophenolate.[16–18] Mycophenolate may be a safer and effective alternative to dapsone as initial therapy for mild to moderate MMP.

Cyclophosphamide was a very successful immunosuppressant, despite being used for the most severe inflammation. This is comparable with previous studies.[4] Unfortunately, adverse events were common, resulting in alteration of therapy in 31%, including 2 life-threatening episodes of pancytopenia and hepatotoxicity. Bladder carcinoma and other malignancies have been reported to occur after pro-

Table 3. Progression of Cicatrization while Receiving Immunosuppressive Therapy

	Total No. Patients	No Progression	Progression	P Value
No. of patients	115	54 (47%)	61 (53%)	
Duration of disease (mos) [mean (range)]	51 (6–300)	43 (6–204)	68 (7–300)	0.0048*
Maximum inflammation grade				
4	22	19 (86%)	3 (14%)	
3	63	31 (49%)	32 (51%)	
2	29	11 (38%)	18 (62%)	
0 or 1	1	0	1 (100%)	0.0007*
Final visual acuity				
Blind	36	21 (58%)	15 (42%)	
Severe visual impairment	8	4 (50%)	4 (50%)	
Visual impairment	18	10 (56%)	8 (44%)	
Normal	53	26 (49%)	27 (51%)	
Predominant outcome†				
Success	86	43 (50%)	43 (50%)	
Qualified success	23	15 (65%)	8 (35%)	
Failure	6	3 (50%)	3 (50%)	
Final inflammation present	31	19 (61%)	12 (39%)	
Continuing therapy at last visit	65	33 (51%)	32 (49%)	
Biopsy positive	43‡	27 (63%)	16 (37%)	
Extraocular disease present	58	35 (60%)	23 (40%)	

Progression defined by an increase in Mondino stage in the worse eye between initial and final visit. Worse eye of each patient selected according to the following criteria: highest Mondino stage, worse inflammation, worse visual acuity, or right eye if both eyes equal.
*Significant at $P<0.01$.
†Outcome present for the greatest duration of the treatment course.
‡Percentage of patients with biopsy results available.

longed use of cyclophosphamide; however, such side effects did not occur in any of our patients. For the above reasons, cyclophosphamide should be used with caution by experienced clinicians and should be reserved for cases where other agents are likely to be ineffective.

Prednisolone or intravenous methylprednisolone was used as an adjunct in 131 treatment episodes and as sole therapy in 6 episodes. It is difficult to establish its value separately from other agents. Its main advantage is that it rapidly controls inflammation during the period while myelosuppressive agents are taking effect. Only 4 adverse events were reported, possibly because of underreporting, but also because of its use in a short course or low dose (≤20 mg/day). Osteoporosis was reported in 2 of the 55 patients (4%) receiving corticosteroids. Prednisolone is not recommended as a sole agent in MMP because of complications from high-dose long-term therapy[25] and recurrence of inflammation when the dose is tapered.[1,8]

Few patients in this study received ciclosporin. Previous investigators have found that 20 (91%) of 22 patients failed to respond to ciclosporin.[1,26,27] Its use in OCP cannot be recommended on the basis of the current evidence.

Combination Therapy and Other Agents

In this study, combination therapy seemed to produce greater successes and fewer failures than single-agent therapy, and the combination of a sulfa, myelosuppressive, and steroid was the most successful (Fig 6 [available at http://aaojournal.org]). There was no apparent increase in the toxicity with this or any other combination of agents.

Several immunomodulatory agents found by others to be efficacious in ocular MMP were used only occasionally in this study. Methotrexate appears to control inflammation in 89% of eyes with mild to moderate disease,[14] but the authors are concerned about irreversible hepatic and pulmonary fibrosis, which can occur with prolonged therapy, and therefore do not use it routinely. Sami et al[13] have shown that intravenous immunoglobulin therapy can be effective in recalcitrant disease; however, it is too expensive and impractical for use as routine therapy. In the authors' institution, it has been used as adjunctive therapy in patients unresponsive to other drugs.

Progression of Disease

Cicatrization. Control of inflammation is not the only goal of therapy in MMP. Although the final outcome in this study was a quiet white eye in 78.5% (175/223) of eyes, there was evidence of progressive cicatrization in 41% (92/223) of eyes. In half of this group of 92 eyes (n = 46), progressive cicatrization was associated with ongoing minimal to mild inflammation that immunosuppressive therapy was unable to eliminate completely, or happened when treatment was discontinued for various reasons, including toxicity. In contrast, the other 46 eyes with progression were reported as being quiet and white. Interestingly, the analysis in Table 3 also indicates that there seemed to be an association between less severe inflammation during the period of follow-up and progression of cicatrization. However, the maximum recorded level of inflammation, on which this analysis is based, was dependent first on the level of inflammation being accurately recorded in the case notes, which may or may not have been accurate on every occasion, and

second on the maximum level of inflammation being present when the patient was examined at follow-up. Furthermore, detection of progression depended on observing an increase in Mondino stage, and this was not recordable for those who started at Mondino stage 4; in contrast, progression was more likely to be detected in those commencing at a lower Mondino stage, and 73% (22/30) of the patients with less severe inflammation (up to grade 2) commenced at Mondino stage 1 or 2. For these reasons, the authors are cautious about making conclusions from this analysis. However, it could suggest that more aggressive treatment given to patients with severe inflammation also more effectively prevented progression of cicatrization, unlike less aggressive, less toxic treatment given for less severe inflammation. Similar progression of cicatrization despite reduction of inflammation has been reported previously.[4,5,12]

The ability to detect progression seems to differ between cicatrization staging systems. The authors found that only 8% of patients progressed according to the Foster staging system, whereas progression was evident in 53% of patients using Mondino staging. Another retrospective review similarly reported 10% progression using Foster staging in a cohort of patients with 90% control of inflammation[7]; the results using the Mondino staging system reflect its increased sensitivity compared with the Foster system. However, in a retrospective study the sensitivity is also limited, as discussed above, to detecting progression in advanced disease.

Visual Loss. The proportion of eyes with visual acuity of 6/60 or less because of OCP doubled from 13% (29/223) to 26% (57/223) by the end of the study. This indicates that although control of inflammation may delay or prevent the onset of surface failure, it is not adequate to eliminate the occurrence of recurrent epithelial defects and microbial keratitis, which lead to irreversible corneal vascularization and scarring.

Reservations about the Study Results

The overall S, QS, and F outcomes for principal agents in this study are subject to bias because the results of both step-up and step-down treatments are combined. The authors attempted separate analysis of step-up and step-down treatments but this was problematic because some treatment changes could not be classified as either step-up or step-down changes. The stepladder approach is a strategy for managing these patients, but whether the strategy is able to be maintained for every single treatment decision depends on the clinical situation, including the need to change therapy because of adverse effects and disease reactivation. A carry-over effect from previous therapy could have influenced the outcome of successive therapies, but by definition S could not be declared until 3 months had passed from the change of therapy, and this was believed to be an adequate washout period to justify analyzing outcomes independent of the effects of previous therapy. The first treatment episode outcomes provide stronger data that are not subject to the effects of previous treatment.

Treatment by indication bias is present, because the severity of inflammation influenced which principal agent was given. However, despite its being used in the most severely inflamed cases, the most successful outcomes were observed with cyclophosphamide. Heterogeneity of the patient group is another shortcoming; interventions such as cataract surgery and fornix reconstruction altered the disease and treatment course in some patients, and may have influenced treatment outcomes. Because this is a retrospective study, the outcomes of treatment at standardized time points after initiation of therapy could not be assessed. This may have led to some bias when assessing final outcomes.

In conclusion, ocular MMP is an uncommon condition, and during their treatment course, patients frequently require several agents either simultaneously or separately. Prospective studies with the complex stratified randomization required to separate treatment groups are unlikely to have sufficient statistical power because numbers in each group will be small. Also, by the time such studies are completed, the treatment paradigms are likely to have altered. These observations illustrate the difficulties in studying MMP, which is a highly variable disease.

Conventional immunosuppressive therapy using a step-up and step-down regimen seems to be partially or completely successful in controlling inflammation in ocular MMP for three quarters of treatment episodes. The extent to which maximum immunosuppressive therapy can be given is limited by its toxicity. Control of inflammation while limiting toxicity may not necessarily correlate with control of cicatrization. A targeted local form of therapy, for local treatment of residual inflammation and cicatrization, given in conjunction with systemic immunosuppression, may improve the management of this difficult disease.

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Appendix 3: IVMP Trial Study Protocol, Participant Information Sheet, Consent Form, Case Report Form, Ethics Committee Approval

A. Study Protocol

PROTOCOL:

A Randomised Controlled Single-masked Trial of Pulsed Intravenous Methylprednisolone for Severe Ocular Mucous Membrane Pemphigoid

Valerie Saw, Saaeha Rauz, Derek Tole, Andrew Dick, John Dart

Background

Mucous membrane pemphigoid (MMP) is a severe subepithelial blistering autoimmune disease affecting the eyes (70%), mucous membranes and skin. Up to 12% of patients with ocular involvement are blind registerable. Systemic immunosuppression is necessary to control the conjunctival inflammation responsible for progressive cicatrisation, ocular surface failure and keratopathy. Oral prednisolone and cyclophosphamide are currently first-line therapy for severe conjunctival inflammation, yet corneal perforation and progressive cicatrisation still occur in 31% of eyes, and disease control is achieved in only 26% by 6 weeks.[1] High dose intravenous immunoglobulin appears effective in recalcitrant ocular MMP, however this requires initially fortnightly infusions of scarce donor blood product for 35 months, and the mean duration to response is 4.5 months.[2,3]

High dose intravenous methylprednisolone pulse therapy may be an effective additional measure to reduce the time to disease control with cyclophosphamide. It may more effectively suppress aggressive inflammation, prevent perforation and perhaps also delay cicatrisation. Its use in ocular MMP is indicated in a report by McCluskey et al but not discussed or formally investigated.[4] In uveitis, pulsed intravenous methylprednisolone (IVMP) reduces inflammation within 1-2 weeks.[5,6] Pulsed intravenous methylprednisolone in rheumatoid arthritis has been shown have similar effects on the synovial membrane to that seen with anti-tumour necrosis factor therapy, yet is more readily available and inexpensive.[7]

The authors wish to test the hypothesis that an initial 3 day course of pulsed intravenous methylprednisolone reduces the time to disease control in patients commencing cyclophosphamide. This study involves a randomized controlled single-masked trial of a short course of pulsed intravenous methylprednisolone in patients with severe inflammation due to active ocular MMP who are commencing oral prednisolone & cyclophosphamide.

I. Aims of the Study

The primary hypothesis is that intravenous methylprednisolone induces more rapid control of ocular inflammation in patients with severe active ocular mucous membrane pemphigoid commencing cyclophosphamide.

II. Plan of the Investigation

A. Basic Organisation

The investigators include

1. Miss Valerie Saw FRANZCO

2. Mr John Dart MA DM FRCS FRCOphth
3. Miss Saaeha Rauz PhD FRCOphth
4. Mr Derek Tole MRCGP FRCOphth
5. Professor Andrew Dick BSc MD FRCP FRCS FRCOphth

The study will be coordinated from Moorfields Eye Hospital and performed at Moorfields Eye Hospital (V Saw, J Dart,), Birmingham and Midland Eye Centre (S Rauz) and Bristol Eye Hospital (A Dick, D Tole).

The study will commence in October 2005 and its likely duration is 36 months.

B. Experimental design and methods The study will be a randomized controlled single-masked clinical trial of an initial 3 day course of pulsed intravenous methylprednisolone versus standard therapy for patients with severe ocular mucous membrane pemphigoid who are commencing oral prednisolone and cyclophosphamide, evaluating the proportion with control of inflammation at 6 weeks as the primary outcome measure.

(a) Study Subjects
Selection criteria: Patients with clinical features consistent with ocular MMP i.e. progressive conjunctival cicatrisation and inflammation, with or without a positive result on direct immunofluorescence microscopy of a conjunctival, mucosal or skin biopsy showing linear basement membrane deposition of IgG, IgA, &/or C3, and/or a positive result on testing for anti-basement membrane zone antibodies.
Inclusion criteria are all patients with bilateral or unilateral 'moderate' (grade 3) or 'severe' (grade 4) ocular inflammation, with or without limbitis (ie oedema and increased vascularity along the limbus). Patients may already be receiving non-cyclophosphamide immunosuppression.
Exclusion criteria include patients currently receiving cyclophosphamide, patients with other causes of progressive conjunctival scarring (drug-induced pemphigoid with negative direct immunofluorescence biopsy, atopic keratoconjunctivitis, Sjogren's syndrome, Stevens Johnson syndrome, chemical injury), active secondary malignancy, HIV infection, pregnancy or breastfeeding.

(b) Study Procedures
Recruitment: Subjects will be identified and contacted by one of the investigators at the 3 eye centres (Moorfields, Birmingham, Bristol) from clinic or following referral to one of these centres for an ophthalmic opinion. The sampling method used is thus multiple cluster (multicentre) sampling. Approximately 10 suitable subjects per year would be expected to be referred to Moorfields Eye Hospital, 5 per year to Birmingham & Midland Eye Centre, 5 per year to Bristol Eye Hospital. The minimum number of required study subjects will be 20, randomized into two groups of 10.

Informed consent: Informed consent will be obtained by one of the investigators at the 3 eye centres. A comprehensive patient information handout will be given to suitable subjects.

Block randomization will be prepared by the Moorfields Eye Hospital R & D statisticians (Dr Catey Bunce & Ms Wen Xing), with stratification by centre.

The intervention: The intervention is intravenous methylprednisolone therapy 1 gram in 250mL of 0.9% saline daily administered over 1 hour for 3 consecutive days, followed by a 3 month tapering course of oral prednisolone 1mg/kg/day, along with commencement of oral cyclophosphamide 1.5 mg/kg/day. Oral prednisolone will be reduced rapidly to 7.5mg by 3 months, then continued at 7.5 to 5mg/d for a further 3 months then ceased.

A second, third or fourth pulse of IVMP may be given at 4 weekly intervals, if the ocular inflammation is not less than grade 2. Thereafter, IVMP may be repeated 3 monthly. Bone densitometry must be performed if more than one course of IVMP is given, and repeated every 6-12 months.

Control treatment: The control treatment is conventional therapy with a 3 month tapering course of oral prednisolone 1mg/kg/day, along with commencement of oral cyclophosphamide 1.5 mg/kg/day.

Oral prednisolone will be reduced rapidly to 7.5mg by 3 months, then continued at 7.5 to 5mg/d for a further 3 months then ceased.

In all patients, cyclophosphamide will be continued as in usual clinical practice for up to 1 year, and replaced with an alternative immunosuppressant where necessary. All patients will be commenced on alendronate 70mg weekly with Calcichew D3 +/- omeprazole 20mg/d until oral steroid doses are <7.5mg/d.

Single masking: Patients and clinical staff will not be masked. One masked observer (an ophthalmologist, trainee ophthalmologist, optometrist or eye nurse) at each eye centre will evaluate will grade conjunctival inflammation and cicatrisation, at 2 weekly intervals for 3 months, 4 weekly for 3 months, then 8 weekly for 6 months, or more frequently depending on clinical necessity. Photographs will also be taken prior to commencing treatment and at each follow-up visit. These photographs will be graded at the Moorfields Eye Hospital Reading Centre

We believe that this short course of intravenous methylprednisolone should show earlier control of inflammation. Achieving better initial control of the inflammation may enable slower-acting cyclophosphamide to more effectively maintain this level of control.

(c) Data to be collected

The primary outcome measure is the proportion of **patients** achieving control of inflammation to grade 0 (nil) or grade 1 (minimal) at 6 weeks. Conjunctival inflammation is graded from grade 0 (nil), grade 1 (minimal), grade 2 (moderate), grade 3 (marked), and grade 4 (severe). Disease is not symmetric and responses between eyes can be very different. The most inflamed eye of each patient will be used to decide the primary outcome. Conjunctival inflammation will be graded at 2 weekly intervals for 3 months, 4 weekly for 3 months, then 8 weekly for 6 months.

For grading, the observers will use a standardized proforma and representative photographs of each grade. [Validation of this grading sheet for assessing conjunctival inflammation will be performed prior, by 3 observers in 10 control patients with different grades of conjunctival inflammation, assessing inter-observer and test-retest reliability]. Results of patient assessment using these proforma and photographs have been published previously by Elder et al.[1,8] This outcome will be qualified as the proportion with attainment of "Complete success", "Qualified success" or "Failure" at 3 months, where these are defined as: "Complete success"- induction of quiescence with a grade 0 or grade 1 eye; Qualified success"- where there is either reduced inflammation, OR additional therapy was needed to control residual inflammation; "Failure"- where there was no response OR therapy had to be withdrawn for unacceptable side effects before an effective response.

The secondary outcome measure is the proportion of patients achieving at 6 weeks a four-fold reduction in titre of circulating antibasement membrane antibodies detected by IIF (indirect immunofluorescence) and/or antibodies to BP180, β4 integrin & laminin 5. Serial titres of circulating anti-basement membrane antibodies have been shown to correlate with disease activity.[9]Serumfor antibasement membrane antibodies will be taken from MEH patients, coinciding with follow-up visits.

Other outcomes to be measured include:

3) Progression of cicatrisation: Tauber grading of ocular cicatrisation by slit lamp exam and using a custom-made fornix measure. This type of fornix measure has been demonstrated to have good inter-rater as well as test-retest reliability.[10]
4) Visual acuity, corneal and lid signs
5) The proportion with adverse effects
6) Both groups of patients will be monitored prior to commencing treatment with BP, blood sugar level, ECG, urinalysis, FBC, U+E, LFTs, +/- CXR if TB risk. Bone densitometry will be performed at the commencement of the trial and at completion. During the 3 day infusions, all patients will have vital observations monitored and daily U+E. Following this, BP, blood sugar, urinalysis, FBC, U+E, LFTs will be checked weekly for 4 weeks, 2 weekly for the second month then monthly. The target lymphocyte count level is 0.5-1.0, aiming for lymphopenia without neutropenia (FBC>3.0 neut >2.0).

7) Fibroblast and T lymphocyte laboratory studies of fresh conjunctival biopsies taken prior to commencing treatment and after treatment when superior bulbar conjunctival inflammation is absent (grade 0) or minimal (grade 1), taking note of the Tauber stage of conjunctival cicatrisation at which the biopsy is taken.

The appointments for grading and follow-up will be organized by the investigators at the 4 eye centres and the Research Coordinator (Suzanne Cabral).

Losses to follow-up will be minimized by contacting the patients when appointments are missed.

Other factors which could influence the outcome include: lack of compliance with oral prednisolone & cyclophosphamide therapy

Procedures to be followed for reporting adverse events:

i) All suspected serious unexpected adverse reactions that are fatal or life-threatening will be recorded and reported as soon as possible to the relevant authorities and to the Ethics Committee (Oxford REC A). Relevant follow-up information will subsequently be communicated within an additional 8 days. All other suspected serious unexpected adverse reactions will be reported to the relevant authorities and the Ethics Committee as soon as possible but within a maximum of 15 days of first knowledge. All investigators will be informed.

ii) Once a year throughout the clinical trial, the study coordinator (Dr Valerie Saw) will provide the MHRA and Ethics Committee with a listing of all suspected serious adverse reactions which have occurred over this period and a report of the subjects' safety.

(d) Sample size calculation & data analysis

As the success rate with conventional treatment is 26% at 6 weeks[1], 9 eyes per treatment group would give 80% power to detect a treatment difference of 70% and 18 eyes per treatment group would give 80% power to detect a treatment difference of 50% at the 5% level. There is no adjustment for loss to follow-up because the study duration is short and the condition is severe.

Baseline characteristics of the treatment groups will be compared to assess the adequacy of randomization. Fisher's exact test will be used to compare success rates in the two groups.

III. Value of this Study

We believe that this pilot randomized controlled trial is the most suitable way to test the hypothesis that pulsed intravenous methylprednisolone achieves better initial control of severe inflammation and improved outcome in ocular pemphigoid.

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B. Participant Information Sheet

Moorfields Eye Hospital NHS
NHS Foundation Trust

City Road
London
EC1V 2PD

Tel: 020 7253 3411
www.moorfields.nhs.uk

Oxfordshire Research Ethics Committee A (05/Q1604/126)

Patient Information Sheet

Intravenous methylprednisolone (IVMP) for severe ocular pemphigoid

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully & discuss it with friends, relatives & your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

1. What is the purpose of the study?

BACKGROUND

You have a condition called pemphigoid, which causes scarring of the front surface of the eye, and sometimes other tissues, due to inflammation. Cyclophosphamide, a chemotherapy-like agent, is needed to control the inflammation when it is severe. Currently, standard treatment is for patients take steroid tablets (prednisolone) with cyclophosphamide. This combination of treatment controls inflammation in only 1/3 eyes by 6 weeks. High dose intravenous steroid (methylprednisolone) which is equivalent to a high dose of oral prednisolone, and frequently used for other diseases such as rheumatoid arthritis, may be an effective additional measure to improve disease control.

AIM

The aim of the study is to determine whether an additional 3-day course of intravenous steroid (methylprednisolone) is better than standard treatment with prednisolone tablets. The study will involve 12 clinic visits and the final visit will be at 12 months. The study is being conducted at 3 eye centres: Birmingham & Midland Eye Centre, Bristol Eye Hospital and Moorfields Eye Hospital.

2. Why have I been chosen to join the study?

We are asking patients with severely active ocular pemphigoid, who would be commencing oral cyclophosphamide and prednisolone to participate in the study. As you have this condition and are suitable we would like to offer you the opportunity to participate. We hope to enrol twenty patients in the study.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. If you do not wish to take part in this study you will not be at a disadvantage and will continue to receive standard treatment.

4. What will happen to me if I take part?

DESIGN

This study will be a randomized trial. This is because sometimes we do not know which way of treating patients is best, such that we need to make comparisons. In this study type, people are put into groups and then compared. A computer, which has no information about the individual, selects the groups-i.e. by chance. Patients in each group then have a different treatment and these are compared. You will have a 50/50 chance of receiving either intravenous methylprednisolone or standard treatment with oral prednisolone tablets.

The trial is single-masked; that is, you and your doctor will know which treatment you received (intravenous or oral), but there will be an independent observer who will not know which treatment you received, who will evaluate your eyes at each visit and assess whether there has been an improvement. This independent observer will either be an eye specialist, trainee eye specialist, optometrist or eye nurse, who has been chosen to assist with this study, for this purpose, at each eye centre.

PROCEDURES

In this study the additional effect of a 3-day course of intravenous steroid (methylprednisolone) will be compared to standard treatment. Participants randomised to intravenous steroid (methylprednisolone) will be admitted to hospital for 3 days for the treatment. Participants randomised to standard treatment will take tablets at home. All participants will be assessed before treatment is started, and at 2 weekly intervals for the first 3 months, then monthly intervals for 3 months and 2 monthly intervals for 6 months. Assessment would be similar to a normal assessment that you would receive if you were not part of the study. Biopsies of the conjunctiva (the transparent skin over the white of the eye) and blood samples are routinely taken as part of the initial assessment of patients with ocular pemphigoid. Blood samples are routinely taken at each clinic visit in patients with ocular pemphigoid, to monitor for toxicity associated with treatment. We would like you to agree to have an additional biopsy of the conjunctiva taken when your eye is less inflamed, and an additional 10mL blood sample taken each time you are having blood taken to monitor for toxicity. The biopsies will be taken using anaesthetic drops and fine instruments. Photographs of your eyes will be taken during the study, but you will not be able to be recognized from such photographs. You will be kept informed of all relevant facts arising as the study progresses.

DURATION AND TERMINATION

The duration of intravenous therapy is 3 days. However, if at any stage we notice that you are having an adverse reaction to the medication it would be ceased. If your condition deteriorates and requires an operative procedure, we may discontinue the treatment if the operation would interfere with the result interpretation.

5. How will the conjunctiva and blood samples be used?

The Oxfordshire Research Ethics Committee A (REC A) has approved the collection of conjunctiva and blood from patients and the use of it by the Institute of Ophthalmology in London for this research. We would like to examine the behaviour of scar cells (fibroblasts) and immune cells (lymphocytes) in the conjunctiva of patients with ocular pemphigoid. These two types of cells are responsible for causing scar tissue formation in ocular pemphigoid. We will be taking two small (4mm x 4mm) pieces of conjunctival tissue, one when your eye is inflamed, and a second piece when your eye is not inflamed. This is to see how treatment changes the activity of the scar cells and immune cells, and to find out what further treatment to control scarring may be possible. Thirteen samples of 10mL blood will be taken over 12 months, to measure antibody levels, which have been shown to correlate with disease activity.

6. Donation of tissue or cells.

The samples will be treated as a gift to the Institute of Ophthalmology, the research partner of Moorfields Eye Hospital in London, who will be responsible for custodianship of the tissue. Although there is no commercial interest sponsoring this research, as the donor you will not have any rights to a share in any profits that might arise from using the sample. Such profits will usually be redirected to further research into eye disease. The samples you provide and data from them may be stored indefinitely. No information in relation to the tissue will be traceable to you, as all personal information will be removed.

7. What do I have to do?

A few lifestyle changes will be necessary. Intravenous methyl**prednisolone** and oral **prednisolone** have similar side effects and require similar lifestyle changes. Prednisolone can induce diabetes (high blood sugar) or worsen diabetic control. You should change your diet to a low sodium, low sugar, high potassium and high calcium diet, and must inform your doctor or nurse if you develop symptoms of frequent urination, increased thirst or hunger, which may be early symptoms of diabetes. Prednisolone can increase your appetite and cause weight gain so it is important to try to limit your intake of high carbohydrate, high fat foods.
Prednisolone may also cause stomach upset and peptic ulceration, and you should inform your doctor if you develop indigestion as this may require treatment with a gastric protection tablet. You should not take non-steroidal anti-inflammatory tablets (e.g. ibuprofen) without prior advice from your GP.

If you are taking >7.5mg a day of prednisolone, you should increase the calcium intake in your diet and take a calcium + vitamin D supplement such as Calcichew D3, 2 tablets daily, to prevent thinning of the bones and fragility fractures. Regular weight-bearing exercise, cessation of smoking and limiting alcohol intake also help prevent bone loss.

These side effects will affect people to different degrees, with some patients having more problems than others. If any of these occur and persist, inform your GP or your eye doctor or nurse.
You should avoid alcohol, except in small quantities (e.g. 1-2 units/glasses of wine or half pints of beer per day.). You can drive and you can play sport. You should continue taking your regular medications.

It is important not to suddenly stop the prednisolone tablets, and to gradually reduce the dose before stopping them. This is because prednisolone tablets affect the body's response to stress and gradually decreasing the dose helps the body cope with this better. It is also important to tell

medical staff that you are taking prednisolone tablets if you need to have a general anaesthetic whilst you are taking the tablets, as they will take this into account when you have the anaesthetic.

8. What is the drug that is being tested?

Intravenous methylprednisolone (and oral prednisolone) belong to a group of medicines called corticosteroids or steroids. Corticosteroids are hormones that are made naturally by your adrenal glands, which are located above your kidneys. They help your body cope with inflammation, allergic reactions and other stresses. Intravenous methylprednisolone (and oral prednisolone) are synthetic corticosteroids, similar to the natural hormone, and work in the same way. They have two main actions: First, they cut down inflammation (pain, redness, swelling and heat) in your body. Their other action is to reduce the activity of the immune system. This is necessary in ocular pemphigoid, which is an autoimmune disease where the immune system is attacking the patient's own body. These medicines are not 'anabolic steroids' which some body builders and athletes misuse.

Intravenous methylprednisolone is equivalent to a high dose of oral prednisolone. The effect of a short (3 day) course of intravenous methylprednisolone will be tested for its ability to rapidly control inflammation. All patients will also take oral prednisolone for 6 weeks, in conjunction with oral cyclophosphamide, as is usual practice for severe ocular pemphigoid.

9. What are the alternatives for treatment?

Traditional treatment for severe ocular pemphigoid is cyclophosphamide and oral prednisolone tablets. Intravenous immunoglobulin may be effective in ocular pemphigoid that is resistant to treatment, although this requires initially fortnightly infusions of donated blood product for an average of 35 months. It is an alternative if cyclophosphamide and prednisolone fail.

10. What are the side effects of taking part?

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. If you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

16. Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/ surgery will have your name and address removed so that you cannot be recognised from it. Your GP will be notified of your participation and this is a precondition of entering the trial.

17. What will happen to the results of the research study?

The results of the study will be published in a number of international scientific journals and in Dr Saw's Doctorate of Philosophy thesis once the study is completed. You will not be identifiable from any published results of the study without your specific consent before publication. We will send you information regarding the results of the study if you request this on the consent from.

16. Who is organizing and funding the research?

This study has been funded by the Special Trustees of Moorfields Eye Hospital and a University College London scholarship. Your doctor will NOT be paid for including you in this study.

17. Who has reviewed the study?

The Oxfordshire Research Ethics Committee A (REC A) has given this study a favourable opinion, which includes extending it to all sites. The local research ethics committees at Moorfields, Birmingham and Bristol have provided the Oxfordshire REC A with an assessment of the facilities

and personnel available. The Oxfordshire Research Ethics Committee A (REC A) reference number is (05/Q1604/126).

18. Contacts for further information

The principal researchers in this study are Mr John Dart and Miss Valerie Saw. If you have any problems or if you would like more information about this study, please do not hesitate to contact one of the persons identified below:

Miss Suzanne Cabral, Research Coordinator Tel: 020 7566 2036 (Moorfields Eye Hospital)

Mr John Dart, Consultant Ophthalmic Surgeon Tel: 020 7566 2320 (Moorfields Eye Hospital)

Miss Valerie Saw, Corneal Fellow Tel: 020 7566 2320 (Moorfields Eye Hospital)

Miss Sanchs Kauz, Consultant Ophthalmic Surgeon Tel: 0121 507 6849 (Birmingham & Midland Eye Centre)

Mr Derek Tole, Consultant Ophthalmic Surgeon Tel: 0117 928 4697 (Bristol Eye Hospital)

Professor Andrew Dick, Consultant Ophthalmic Surgeon Tel: 0117 928 4653 (Bristol Eye Hospital)

After hours, you can contact the resident on duty at Moorfields Eye Hospital (020 7253 3411), Birmingham & Midland Eye Centre (0121 554 3801) or Bristol Eye Hospital (0117 923 0060) to obtain advice. You will be given a copy of this information sheet and a signed consent form to keep. The original will be placed in your notes.

THANK YOU

C. Participant Consent Form

Moorfields Eye Hospital NHS
NHS Foundation Trust

Oxfordshire Research Ethics Committee A (05/Q1604/126)
Patient Identification Number for this trial:

City Road
London
EC1V 2PD

Tel: 020 7253 3411
www.moorfields.nhs.uk

CONSENT FORM

Title of Project: Intravenous methylprednisolone for severe ocular pemphigoid

Name of Researchers: Mr John Dart, Miss Valerie Saw, Miss Saeeha Rauz, Professor Andrew Dick, Mr Derek Tole, Dr Virginia Calder, Dr Julie Daniels, Professor Stephen Moss

1. I confirm that I have read the attached information sheet on the above project, dated 23.08.2005 (version 3) for the above study and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that my GP will be informed of my participation in the above project. ☐
4. I agree to give samples of conjunctiva and blood for research in the above project. I understand how the samples will be collected, that giving a sample for this research is voluntary and I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐
5. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐
6. I agree that the sample I have given and the information gathered about me can be looked after and stored indefinitely at the Institute of Ophthalmology for use in future projects, as described in the attached information sheet. I understand that researchers other than those listed above may carry out some of these projects. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Would you like to be sent information about the results of the research? YES ☐ NO ☐

Thank you for giving samples for research
1 for patient; 1 for researcher; 1 to be kept with hospital notes

IVMP Consent Form Version 3, 23.08.05

Chairman: Sir Thomas Boyd-Carpenter
Chief Executive: Ian Balmer
Patron: Her Majesty The Queen

D. Case Report Form

Oxfordshire Research Ethics Committee A (05/Q1604/126) (JD/VS IVMP trial version 6:2005)

Systemic Immunosuppression Chart

Name: **Weight:** **MRN:** **Study Number:** ☐☐

Visit	DATE	DRUG	CUURENT DOSE	Planned doses till next visit	COMMENTS
Enrolment		IVMP			
		Cyclophosphamide			
		Prednisolone			
Week 2		Prednisolone			
Week 4		Prednisolone			
Week 6		Prednisolone			
Week 8		Prednisolone			
Week 10		Prednisolone			
Week12		Prednisolone (5- 7.5mg)			
Week 16		Prednisolone			
Week 20		Prednisolone			
Week 24		Prednisolone			
Week 32		Prednisolone			
Week 40		Prednisolone			
Week 48		Prednisolone			
Week 52		Prednisolone			

RESULTS

Visit	DATE	BP	BSL	Urine	Hb (13.0-17.0)	WBC (4.0-11.0)	Neut (1.5-7.0)	Lymph (1.2-3.5)	Plat (150-400)	Na (135-145)	K (3.5-5.0)	Urea (1.7-8.3)	Creat (59-104)	Bil (0-16)	Alk Phos (35-129)	ALT (4-59)	γGT (4–72)	IIF titre
Enrolment																		
Wk 1 (GP)																		
Week 2																		
Wk 3 (GP)																		
Week 4																		
Week 6																		
Week 8																		
Week 10																		
Week 12																		
Week 16																		
Week 20																		
Week 24																		
Week 32																		
Week 40																		
Week 48																		
Week 52																		

BASELINE TESTS

All patients: FBC, U+E, LFTs, blood sugar, lipids, and BP.
Steroids: Consider CXR (exclude TB) & ECG (prior to methylprednisolone pulse)
Cyclophosphamide: Urinalysis. **Dapsone**: G6PD & FBC. **Azathioprine**: TPMT (thiopurine methyltransferase) level

Only complete shaded boxes if results abnormal

Visit	DATE	TEST (Immunofluorescence, CXR, ECG, Bone densitometry)	RESULT

IVMP for ocular pemphigoid trial **Enrolment Visit**

Name: **EYE ENROLLED** (please circle): **OD** **OS**

Sex: **STUDY NUMBER:**

DOB: Enrolment date:

Hospital Number: Visit date:

Study Centre: *Oxfordshire REC A (05/Q1604/126)*

Date of diagnosis of MMP: Age at diagnosis: ☐☐ yrs Duration since diagnosis: ☐☐ mths Presenting symptoms of MMP:

Type of MMP ☐ If 2, list causative medication & eye(s) involved……

1= putative autoimmune 2= drug-induced 3= linear IgA, dermatitis herpetiformis 4= Stevens-Johnson syndrome 5= other

Biopsy results:

Site	Date	DIF report	Histology

Indirect IF results: Date Report & Titre

Extraocular manifestations ☐

0= nil 1=yes 2= unknown

If yes, specify sites……

Associated autoimmune disease (eg Sjogren's, RA, atopy) ☐

0=nil 1=yes 2=unknown If yes, specify……

Immunosuppressive Treatment (previous & current)

Date commenced	Medication	Date ceased	Reason for change

Other current medications (Oral and Topical)

Name	Dose	Frequency	Indication

Contact lens wear (current) ☐

0= nil 1= soft 2= hard 3= scleral 4= failed 5= unknown

Corneal graft ☐

0= nil 1= penetrating 2= lamellar 3= limbal stem cell 4= unknown

Lid surgery *(list more than one if applicable)* **Upper** ☐ ……

0= nil 1= cryotherapy 2= electrolysis

3= anterior lamellar reposition (or Jones retractor plication) & grey line split

*4= fornix reconstruction (**specify TYPE** eg MMG, HP, ear cartilage, amnion)* **Lower** ☐ ……

5= ectropion surgery 6= other lid surgery (specify)

ALL FIELDS MUST BE COMPLETED

Other Past Ocular History

Diagnosis	Date of Diagnosis	Treatment

Past Medical History

Diagnosis	Date of Diagnosis	Treatment

CURRENT SYMPTOMS IN THIS EYE

0= no 1= mild 2= moderate 3= severe but not interfering with activities 4= very severe, interfering with activities

Photophobia……	
Gritty ……	
Painful or sore ……	
Blurred vision……	
Poor vision……	

CURRENT SYSTEMIC SYMPTOMS

Extraocular MMP ……

Other……

MASKED OBSERVER TO EXAMINE **this eye** NOW and complete **full** Assessment Sheet

Investigator to identify Enrolled Eye for Observer, on Masked Observer Assessment Sheet

STUDY INVESTIGATOR then to:

1. Contact Wen Xing 020 7566 2284 regarding randomisation outcome (IVMP versus conventional)
2. Conduct baseline tests: FBC, U&E, LFTs, and take blood (clotted x1, heparinx1) for research
   Organise Bone Densitometry, CXR, ECG
   Complete the Immunosupppression Chart details
3. Photographs
4. Take 4mm conjunctival biopsy for research ± immunofluorescence (± buccal)
5. Admit for IVMP if randomised to this. Dispense medication if randomised to conventional therapy
6. Send GP letter requesting week 1, week 3 blood tests
7. Give next visit date

Comments:

ALL FIELDS MUST BE COMPLETED

IVMP for ocular pemphigoid trial **IVMP admission**

Name:	**EYE ENROLLED** (please circle): **OD** **OS**
Sex:	**STUDY NUMBER**:
DOB:	Enrolment date:
Hospital Number:	Visit date:
Study Centre:	*Oxfordshire REC A (05/Q1604/126)*

IVMP regime

Date	Dose given	Comments

ALL FIELDS MUST BE COMPLETED

IVMP for ocular pemphigoid trial **Week 2 Visit**

Name: **EYE ENROLLED** (please circle): **OD** **OS**

Sex: **STUDY NUMBER**:

DOB: Enrolment date:

Hospital Number: Visit date:

Study Centre: *Oxfordshire REC A (05/Q1604/126)*

Current Medications (Oral and Topical)

Name	Dose	Frequency

CURRENT SYMPTOMS IN THIS EYE

0= no 1= mild 2= moderate 3= severe but not interfering with activities 4= very severe, interfering with activities

Photophobia..... ☐

Gritty ..... ☐

Painful or sore ..... ☐

Blurred vision..... ☐

Poor vision..... ☐

CURRENT SYSTEMIC SYMPTOMS.....

Any Adverse Effects

Overall acceptability of current medication ☐

1= very good 2= good 3= neither good nor bad 4= bad 5= very bad

MASKED OBSERVER TO EXAMINE **this eye** NOW

Investigator identifies enrolled eye on Observer Sheet

IS THE ENROLLED EYE AT ENDPOINT?

If **YES**: 1. Conjunctival biopsy and blood (clotted x1, heparin x1) to be taken and sent to Valerie Saw

If **NO or YES**:

1. Obtain blood results from previous visit, complete Immunosuppression Chart, order today's blood tests, plan immunosuppression regime
2. Photographs
3. Scripts for planned immunosuppression regime
4. Arrange next visit date

Comments: (eg microbial keratitis, PED, other medical or surgical treatment independent of trial)

ALL FIELDS MUST BE COMPLETED

IVMP for ocular pemphigoid trial **Week 6 Visit**

Name: **EYE ENROLLED** (please circle): **OD** **OS**

Sex: **STUDY NUMBER**:

DOB: Enrolment date:

Hospital Number: Visit date:

Study Centre: *Oxfordshire REC A (05/Q1604/126)*

Current Medications (Oral and Topical)

Name	Dose	Frequency

CURRENT SYMPTOMS IN THIS EYE

0= no 1= mild 2= moderate 3= severe but not interfering with activities 4= very severe, interfering with activities

Photophobia..... ☐

Gritty ..... ☐

Painful or sore ..... ☐

Blurred vision..... ☐

Poor vision..... ☐

CURRENT SYSTEMIC SYMPTOMS.....

Any Adverse Effects

Overall acceptability of current medication ☐

1= very good 2= good 3= neither good nor bad 4= bad 5= very bad

MASKED OBSERVER TO EXAMINE **this eye** NOW

Investigator identifies enrolled eye on Observer Sheet

IS THE ENROLLED EYE AT ENDPOINT?

If **YES**: 1. Conjunctival biopsy and blood (clotted x1, heparin x1) to be taken and sent to Valerie Saw

If **NO or YES**:

1. Obtain blood results from previous visit, complete Immunosuppression Chart, order today's blood tests, plan immunosuppression regime
2. Photographs
3. Scripts for planned immunosuppression regime
4. Arrange next visit date

IS THERE A FOUR-FOLD REDUCTION IN IIF TITRE? Yes/ No/ Unassessable (circle)

Comments: (eg microbial keratitis, PED, other medical or surgical treatment independent of trial)

ALL FIELDS MUST BE COMPLETED

E. Ethics Committee Approval

Oxfordshire REC A
Room 13, Manor House
John Radcliffe Hospital
Headley Way, Headington
Oxford
OX3 9DZ

Telephone: 01865 222758
Facsimile: 01865 222699
Email: gordon.riddell@orh.nhs.uk

29 September 2005

Mr John Dart
Consultant Ophthalmic Surgeon
Moorfields Eye Hospital NHS Foundation Trust
162 City Road
London
EC1V 2PD

Dear Mr Dart

Full title of study:	**A Randomised Trial of Pulsed Intravenous Methylprednisolone for Severe Ocular Pemphigoid**
REC reference number:	**05/Q1604/126**
Protocol number:	**2**
EudraCT number:	**2005-002391-14**

Thank you for your letter of 23 August 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Please note there is a typo on Page 2, paragraph 1 of the Patient Information Sheet. It should read "patients to take steroid tablets"

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		08 July 2005
Investigator CV		01 May 2005
Protocol	2	28 June 2005
Covering Letter		08 July 2005
Covering Letter		23 August 2005
Covering Letter		31 August 2005
Letter from Sponsor		29 June 2005
GP/Consultant Information Sheets	3	23 August 2005
Participant Information Sheet	3	23 August 2005
Participant Information Sheet – Control	2	23 August 2005
Participant Consent Form – Control	2	23 August 2005
Participant Consent Form	3	23 August 2005
Response to Request for Further Information		23 August 2005
Systemic Immunosuppression Chart	JD/VS 5:2005	
Letter from funders - UCL		25 May 2005
Letter from funders - Special trustees		11 July 2005
Systemic Immunosuppression Chart		08 July 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q1604/126	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Best wishes

Yours sincerely

Dr Brian Shine
Chair

Appendix 4: Upper Conjunctival Fornix Depth Study Protocol, Participant Information Sheet, Consent Form, Ethics Committee Approval, Preliminary data

A. Study Protocol

Upper and Lower Conjunctival Fornix Depth- A method of objective assessment: Protocol v2, 16.1.2007

Claire Daniel, Valerie Saw, Scott Hau, David Carpenter, Catey Bunce, Wen Xing, John Dart

Introduction

Measurement of conjunctival cicatrisation in ocular mucous membrane pemphigoid, according to Mondino, Tauber and Rowsey, focuses only on in inferior fornix depth.[1-3] Progression of cicatrisation by a reduction in superior fornix depth is often overlooked. Schwab and coauthors designed a custom-made inferior fornix measurement instrument, which was shown to produce reliable, reproducible results. Normal inferior fornix depth has been shown to be 10mm in those aged over 80, and 11mm in those aged 60 or less.[4] However little attention has been given to the normal upper fornix depth.

Aim

This study will be undertaken to develop, as an extension of the work of Schwab and colleagues, an objective technique for measuring upper fornix depth. The purpose of this is to aid detection of and measure progression in cases of suspected cicatrising conjunctivitis, and enable measurement of progression of upper fornix cicatrisation in ocular mucous membrane pemphigoid.

Materials & Methods

Instrument

We have designed an adaptation of the Schwab instrument to measure the upper conjunctival fornix. The same instrument can be used to measure the inferior conjunctival fornix. Whilst the Schwab instrument is made of aluminium, our instrument is made of plastic, and similarly biconcave and designed to fit between the globe and the upper lid. A

standardized scale is inscribed on the outer surface with 2mm black line gradations, and red lines at 10 and 20mm. The maximum number of gradations on the instrument is 22 to 24. A longer instrument, with more gradations, could be made where necessary, for measurement of deeper fornices such as in patients with giant fornix syndrome. The measurements are performed without the use of a slit lamp.

After administration of topical anaesthetic, the patient is asked to look down and the fornix measure is gently inserted upwards vertically in the upper cul-de-sac, until resistance is felt, taking care to avoid stretching the cul-de-sac. In the same manner as with the Schwab instrument, where the upper eyelid margin rests on the scale inscribed on the fornix measure is taken as the measurement of the upper fornix. If the lid margin falls between two marks, an additional 0.5mm is added to the total. The measurement of the upper fornix is taken in downgaze as it uncomfortable for the patient to attempt primary gaze with the instrument in place. The measurement of the lower fornix is taken in upgaze for similar reasons.

The instrument is disinfected between subjects by soaking in hypochlorite for 5 minutes then washing off the hypochlorite with sterile saline (see sterilization protocol). The instrument does not come in contact with the cornea.

Subjects

Both eyes of 120 patients in each age group (50-59, 60-69, 70-79, 80+) will be collected for the study. “Normal” subjects will be selected from the outpatient clinics and emergency department of Moorfields Eye Hospital.

A separate subset of 25 normal subjects will be measured by 2 separate observers to calculate interobserver error. These subjects will be measured again at a later time by one of the observers in a masked fashion to calculate intraobserver error.

Inclusion criteria for “normal” subjects;

Not using any topical medication apart from lubricants.

No history of ocular surface disease or ophthalmic surgery other than small incision phaco-emulsification.

Exclusion criteria;

Patients with ptosis, ptosis surgery or giant fornix syndrome

Patients with a history of ocular allergy, rosacea..

Patients with conjunctival scarring (post adenoviral conjunctivitis or membranous bacterial conjunctivitis, chemical or thermal burns, Stevens Johnson syndrome, mucous membrane pemphigoid, atopic keratoconjunctivitis, trachoma, radiotherapy).

Results/ Statistical analysis

Age stratified measurements of normal subjects will be compared. A Bland and Altman plot will be used to compare the variance of the study population within the age ranges and between the chosen age groups. Analysis of inter and intraobserver error will be performed.

Value of this study: We have designed a modified fornix measure which enables objective measurement of both upper and lower conjunctival fornix depth. Establishing what the normal upper fornix depth will assist in evaluating cases of suspected cicatrising conjunctivitis and will aid monitoring progression in these cases and in other conditions with conjunctival scarring.

References

1. Mondino BJ, Brown SI. Ocular cicatricial pemphigoid. *Ophthalmology* 1981;88:95-100.
2. Tauber J, Jabbur N, Foster CS. Improved detection of disease progression in ocular cicatricial pemphigoid. *Cornea* 1992;11(5): 446-451.
3. Rowsey JJ, Macias-Rodriguez Y, Cukrowski C. A new method for measuring progression in patients with ocular cicatricial pemphigoid. *Arch Ophthalmol* 2004; 122:179-184.
4. Schwab IR, Linberg JV, Gioia VM, Benson WH, Chao GM. Foreshortening of the inferior conjunctival fornix associated with chronic glaucoma medications. *Ophthalmology* 1992;99:197-202.

B. Participant Information Sheet

Moorfields Eye Hospital NHS
NHS Foundation Trust
Patron: Her Majesty The Queen

Chairman
Sir Thomas Boyd-Carpenter

Chief Executive
I.A.J Balmer

City Road
London
EC1V 2PD

Tel 020 7253 3411
Minicom 020 7566 2279

Patient Information Sheet

1. Study Title
Upper and Lower Conjunctival Fornix Depth- A method of objective assessment

2. Invitation
You are invited to take part in this research study. Before you decide it is important for you to understand why the research is being done and what's involved. Please take time to read the following information carefully and discuss it with your family, friends, hospital specialist or GP if you wish.

What is the purpose of the study?
We would like you to agree to have some measurements of the eye done to find out the normal range of the depth of the upper and lower lids. Some eye problems end up with severe shortening of the space between the upper and lower lids. No studies so far have provided normal values for the depth of the upper and lower lids. It is our aim to measure normal variations in this measurement so that we can compare these to measurements in people with eye diseases.

Why you are being invited to take part?
You are being invited to take part because your eyes have no previous ocular problems and would supply us with a normal range of measurements for our study.

3. What does the study involve?
We will be putting some anaesthetic drops into the eye and we will be briefly placing a small measure in the upper and lower lids.

4. What will the results be used for?
The results of this study will be used to provide a range of normal values of the upper and lower lid space. There are scarring conditions of the eye that can result in severe shortening of the upper and lower lid space, which can lead to drying and infection. This will help us detect such eye disease earlier and help monitor progression of scarring eye disease in the future. There are no companies funding this research. None of the information will be traceable to you, as all personal information will be removed. The measurement may be shared with other researchers, if the Local Research Ethics Committee approves this.

5. What do I have to do?
You don't have to do anything, apart from to give your consent. Your doctor will do the measurements after they have put some anaesthetic drops in your eye. The anaesthetic drops will wear off in approximately half an hour.

6. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

7. What are the disadvantages and risks of taking part?
The only difference to your treatment is that you will have an anaesthetic drop in your eyes. This will allow us to put the measure in the correct place. .

8. What are the possible benefits of taking part?
There will be no benefit for any participants in this study, but taking part and allowing us to use your measurements will allow us to provide better care for those people with eye problems. We will be able to compare their measurements to yours and this hopefully will allow us to detect problems at a much earlier stage.

9. What if something goes wrong?
It is difficult to foresee any harm resulting from this study. If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action, but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

10. Will my taking part in this study be kept confidential?
The measurements will be kept separate from any personal information you provide us with. All data will be anonymous and coded to ensure confidentiality. If you have no objections, we will inform your GP that you have been asked to take part in this study.

11. What will happen to the results of the research study?
The results of this study are likely to be published in a number of scientific journals. While sometimes this attracts attention from journalists, you will never be identified in any report or publication.

12. Who has reviewed the study?
The Moorfields Eye Hospital Research Ethics Committee has reviewed this study.

Contacts for further information
Miss Suzanne Cabral, Research Co-ordinator, telephone number 020 7566 2036

Miss Claire Daniel, SpR Moorfields Eye Hospital, telephone number 020 7253 3411

Miss Valerie Saw, Cornea and External Disease Research Fellow, 020 7566 2320

THANK YOU

You may keep a copy of this information sheet and the consent form you have signed.

C. Consent form

Moorfields Eye Hospital NHS
NHS Foundation Trust
Patron: Her Majesty The Queen

Chairman
Sir Thomas Boyd-Carpenter

Chief Executive
I.A.J Balmer

City Road
London
EC1V 2PD

Tel 020 7253 3411
Minicom 020 7566 2279

CONSENT FORM

Title of project: **Upper and lower conjunctival fornix depth- A method of objective assessment**

Name of researcher: Miss Claire Daniel, Miss Valerie Saw, Mr Scott Hau, Mr John Dart

1. I have read the attached information sheet on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved. ☐

2. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

3. I understand that my GP will be informed of my participation in this study. ☐

4. I know how to contact the research team if I need to: Miss Suzanne Cabral, Research Co-ordinator, telephone number 020 7566 2036. ☐

.....	.....	.....
Name of patient (BLOCK CAPITALS)	Date	Signature
.....	.....	.....
Name of person taking consent	Date	Signature
.....	.....	.....
Name of researcher	Date	Signature

Would you like to be sent information about the progress of this project? YES NO

Thank you very much for helping with research on eye disease.

One copy for patient, one copy for notes, one copy for researcher

Consent Form Version 2 16/1/2007

D. Ethics Committee Approval

Moorfields & Whittington Local Research Ethics Committee
South House, Block A
Royal Free Hospital
Pond Street
London
NW3 2QG

Telephone: 020 7794 0552
Facsimile: 020 7794 0714

Mr John Dart
Consultant Ophthalmic Surgeon
Moorfields Eye Hospital
City Road
London
EC1V 2PD

09 February 2007

Dear Mr Dart

Full title of study: **Upper Conjunctival Fornix Depth: A method of objective assessment**
REC reference number: **06/Q0504/104**

Thank you for your letter of 01 February 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	1	02 November 2006
Investigator CV		31 May 2006
Protocol	2	16 January 2007
Covering Letter		03 November 2006
Letter from Sponsor		03 November 2006
GP/Consultant Information Sheets	1	16 January 2007
Participant Information Sheet: Inter and intraobserver	1	16 January 2007

Participant Information Sheet	2	16 January 2007
Participant Consent Form	2	16 January 2007
Response to Request for Further Information		01 February 2007
Response to Request for Further Information		01 February 2007
Sterilisation Protocol		06 February 2007
Principal investigator CV		02 November 2006

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0504/104	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp **John Farrell**
Chair

Email: katherine.clark@royalfree.nhs.uk

Enclosures: *Standard approval*
Site approval form

Copy to: R&D Department, Moorfields Eye Hospital NHS Foundation Trust

E. Preliminary Results

	Age (years)				
	50-59	**60-69**	**70-79**	**80+**	**All**
	n = 28	n = 36	n = 41	n = 23	n=127
Mean upper conjunctival fornix depth (mm)	15.5	15.2	15.4	14.6	15.2
Standard deviation	1.9	1.3	1.7	1.6	1.6
Range	12 to 20	12 to 18	12 to 20	12 to 18	12 to 20

Appendix 5: Pemphigoid Laboratory Study Protocol, Participant Information Sheet, Consent Form

A. Study Protocol

PROTOCOL:

Conjunctival scarring in Ocular Mucous Membrane Pemphigoid

Valerie Saw, Virginia Calder, Julie Daniels, John Dart

Background

Mucous membrane pemphigoid (MMP) is an autoimmune disease associated with autoantibodies to the basement membrane. Conventional therapy for MMP is systemic immunosuppression.[1] Progressive cicatrisation can occur despite control of inflammation with immunosuppressants.[2;3] Acute inflammation correlating with rapid conjunctival shrinkage in some patients provides the rationale for use of systemic immunosuppression to prevent cicatrisation, yet other patients develop slowly progressive cicatrisation despite minimal inflammation. In the latter patients, further immunosuppression is unnecessary but adjunctive anti-fibrosis therapy would be beneficial. How well immunosuppressive therapy inhibits fibrogenesis in MMP has not been elucidated, and it may be that anti-fibrosis therapy in addition to immunosuppression at the time of acute inflammation would more effectively prevent the aggressive cicatrisation which leads to blindness. Previous studies at the Institute of Ophthalmology have demonstrated elevated levels of T lymphocytes and transforming growth factor TGF-β1 and β3 in the conjunctiva in acute phase MMP.[4;5] However, fibrogenic cytokines are absent during the chronic progressive phase, leading to the hypothesis that MMP fibroblasts remain functionally abnormal after withdrawal of cytokine influences. Conjunctival fibroblasts in MMP appear to be abnormally hyperproliferative.[6-8]

We believe that characterisation of the fibroblasts in pemphigoid and investigation of the mechanism of fibrosis in this condition may lead to additional targeted therapy to improve control of the fibrotic aspect of the disease.

Aims of the Study

The primary hypothesis is that immunosuppressive therapy does not affect the conjunctival fibroblasts in MMP, which are functionally abnormal.
The secondary hypothesis is that T lymphocyte-derived cytokines and T cell- fibroblast cross talk play a role in fibrogenesis in MMP.

Plan of the investigation

A. Basic Organisation
The investigators include:

1. Dr Valerie Saw FRANZCO
2. Dr Virginia Calder BSc(Hons) PhD
3. Dr Julie Daniels BSc(Hons) PhD
4. Mr John Dart DM FRCS FRCOphth

Patients will be enrolled from the External Disease clinics at Moorfields Eye Hospital (Dr Saw, Mr Dart) and the laboratory research will be performed at the Institute of Ophthalmology (Dr Saw, Dr Calder, Dr Daniels). The study will commence in February 2006 and its likely duration is 30 months.

B. Study Design & Methods:
Conjunctival biopsies will be taken from ocular pemphigoid patients at two stages in the course of the disease: first when the eyes are acutely inflamed, and secondly when the eyes white and inflammation has been controlled with immunosuppressive therapy. Fibroblast and T lymphocyte cultures will be derived from these biopsies, and immunohistochemistry will be performed on the conjunctival tissue.

(a) Study Subjects
Selection criteria: Patients with clinical features consistent with ocular MMP i.e. progressive conjunctival cicatrisation and inflammation, with or without a positive result on direct

immunofluorescence microscopy of a conjunctival, mucosal or skin biopsy showing linear basement membrane deposition of IgG, IgA, &/or C3, and/or a positive result on testing for anti-basement membrane zone antibodies.

Inclusion criteria are:

(a) patients with bilateral or unilateral active conjunctival inflammation due to MMP
(b) patients with bilateral or unilateral MMP where inflammation has been controlled with immunosuppressive therapy

Suitable patients in group (a) may also fall into group (b) after treatment, and these patients will be asked to consent to two conjunctival biopsies. Suitable patients may also remain in group (a) or in group (b), and these patients will be asked to consent to one conjunctival biopsy only.

Exclusion criteria include patients with other causes of progressive conjunctival scarring (drug-induced pemphigoid with negative direct immunofluorescence biopsy, atopic keratoconjunctivitis, Sjogren's syndrome, Stevens Johnson syndrome, chemical injury), active secondary malignancy, HIV infection.

(b) Study Procedures

Recruitment: Study subjects will be identified and referred to the study from the External Disease clinics at Moorfields Eye Hospital. The minimum number of required study patients is 10; five from group (a) and five from group (b).

Informed consent: Informed consent will be obtained by Dr Saw. A comprehensive patient information handout will be given to suitable subjects.

Conjunctival biopsies and blood samples: A 4mm x 4mm bulbar conjunctival biopsy will be taken under topical anaesthesia. Performing a conjunctival biopsy is part of routine initial management of ocular MMP. 10mL blood will also be collected when each biopsy is taken, as a source of T lymphocyte feeders.

Confidentiality: Biopsies and blood samples will be labelled with a study identification number and any information about the biopsies and blood samples will have the patient's name and address removed so that they cannot be recognised from it.

(c) Laboratory research to be performed:

Obtaining biopsies before and after control of inflammation with immunosuppressive treatment will provide a unique opportunity to investigate the effects of treatment on the conjunctiva and its cells. It will also provide the ideal setting in which to investigate the behaviour of T lymphocytes and fibroblasts during active MMP.

Half of the conjunctival specimen will be used for fibroblast cultures, and half for immunohistochemistry and T lymphocyte studies. Tissue for immunohistochemistry will be embedded in glycol methacrylate (GMA) resin.

a) Fibroblast cultures and functional assays-The biology of the fibroblast in MMP has not been fully explored. 5 key aspects of fibroblast function will be evaluated[9]: proliferation, migration, collagen production, matrix contraction, metalloproteinase production. Microarray analysis of fibroblast gene expression will also provide information regarding up and down-regulated proto-oncogenes, growth factors and transcription factors.

The behaviour of conjunctival fibroblasts from biopsies obtained before and after control of inflammation with immunosuppressive therapy will be compared, to investigate the effects of immunosuppressive treatment on fibroblast function. The effect of T lymphocyte-derived cytokines detected in MMP (IL-4, IL-5, IFNγ, TGFβ) on fibroblast function will also be evaluated. Addition of neutralizing monoclonal antibodies specific for these cytokines or their receptors, into the fibroblast assays, will also be investigated.

b) T lymphocyte studies- T lymphocytes expanded in culture from the conjunctival biopsies will be immunophenotyped by flow cytometry. Cytokine production will be quantified by flow cytometry-based multiplex assays,[10] investigating the presence of T_H2 (IL-4, IL-5, IL-13) and T_H1 (IFNγ, TGFβ, IL-2, TNFα) cytokines, to further characterize the T lymphocyte cytokine pattern in ocular MMP. Cytokine profiles from biopsies obtained before and after control of inflammation with immunosuppressive therapy will be compared. The effect of these cytokines on in vitro fibroblast characteristics will then be evaluated. The effect of immunosuppressive therapy on fibroblast expression of costimulatory molecules (CD86, CD40, CD154) will be evaluated by immunohistochemistry & flow cytometry.

c) Whole conjunctival tissue studies- Whole conjunctival tissue will be studied by immunohistochemistry[11] to determine the overall effects of immunosuppressive treatment on the intensity and distribution of staining for: **i) Fibroblast activation markers**- HLA-DR, ICAM-1, costimulatory molecules (CD 86, CD40, CD154) **ii) $CD25^+$ T lymphocytes iii) IL-13 and TNF-α expression. iv) CCL3 and CCL2 chemokines v) Evidence for the presence of Treg cells** in MMP conjunctiva will be investigated by immunostaining for transcription factor Foxp3 mRNA expression,[12] as well as by Western blotting. Further functional assays for Treg will be performed by determining their ability to inhibit T lymphocyte proliferation when added exogenously to proliferation assays.

Finally, time-permitting, **the effect of co-culture of fibroblasts with MMP-derived T lymphocytes** from the same donors will be evaluated to determine the effects on fibroblast activation, collagen production, and expression of activation markers following exposure to T cells.

Statistical analysis: Laboratory data will be analysed using the MinitabTM programme, using Mann-Whitney tests to compare non-parametric values, and SPSS for Windows for one-way analysis of variance (ANOVA).

References

(1) Foster CS. Cicatricial pemphigoid. Trans Am Ophthalmol Soc 1986; 84:527-663.
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Moorfields & Whittington LREC 06/Q0504/20

Patient Information Sheet

For Active Pemphigoid Patients
Conjunctival scarring in ocular pemphigoid

1. Study Title

Conjunctival scarring in ocular pemphigoid

2. Invitation
You are invited to take part in this research study. Before you decide it is important for you to understand why the research is being done and what's involved. Please take time to read the following information carefully and discuss it with your family, friends, hospital specialist or GP if you wish.

3. What is the purpose of the study?
We would like you to agree to have some conjunctiva taken from the eye. This is a transparent skin over the white of the eye.

Currently, standard treatment for patients with ocular pemphigoid is to take chemotherapy-like medication to control inflammation. It is not known how well this medication stops the formation of scar tissue. Laboratory research conducted on biopsies from patients suffering from pemphigoid may discover new treatment targeted at preventing the scar tissue formation, which is ultimately responsible for blindness.

The aim of the study is to see how treatment changes the activity of the scar cells and immune cells, and to find out what further treatment to control scarring may be possible.

4. Why you are being invited to take part
You are being invited to take part because you have ocular pemphigoid.

5. How will the samples be used?
The local Research Ethics Committee has approved the collection of conjunctiva and blood samples from patients and the use of it by the Institute of Ophthalmology, the research partner of Moorfields Eye Hospital, in this research. We will examine the behaviour of immune cells (lymphocytes) and scar cells (fibroblasts) in the conjunctiva. This may lead to discovery of new treatment for ocular pemphigoid. The blood samples will provide immune cells (lymphocytes) which are needed to enable the immune cells from the conjunctiva to survive in the laboratory.

Taking a conjunctival biopsy is part of the routine assessment of ocular pemphigoid. We would like to use a small piece of this tissue (about 4mm x 4mm) for research. We would like to take two different types of biopsies: one type from patients who have active disease, and the second type from patients where the disease has been controlled with medication. In some patients, the duration of the

study will allow us to follow them from the beginning when their eyes are inflamed and active, to the time when the disease is controlled with medication- in these patients, we would like to take a second biopsy when their eyes are white and the disease is under control. In other patients, we will only request biopsies when the eyes are active and inflamed, or alternatively when the eyes are white after taking medication. We will be comparing the differences between the two types of biopsies. In your case, at the moment the disease is active and your eyes are inflamed.

4. Donation of tissue or cells.
The samples will be treated as a gift to the Institute of Ophthalmology, who will be responsible for custodianship of the tissue. As the donor, you will not have any rights to a share in any profits that might arise from using the sample.

5. Who will sponsor this research?
This study has been funded by Action Medical Research, the Special Trustees of Moorfields Eye Hospital and a University College London scholarship. Your doctor will NOT be paid for including you in this study. There are no commercial interests sponsoring this research.

6. What will happen to me if I take part?
Your eye will be anaesthetised using drops and using a cotton bud soaked with anaesthetic, placed over the area where the biopsy will be taken. A small piece of conjunctiva will be taken with small scissors and forceps and placed in a specimen pot. The pot will be collected by the researcher, Dr Saw, from the Institute of Ophthalmology. We will also take 10ml blood each time a biopsy is taken.

The samples you provide and data from them will be stored for up to 10 years. Future use of the samples will only occur in studies which have ethics committee approval. We may need to analyse the expression of genes in the cells from the samples, but no personal information will be provided with the sample so the results will not be linked to you. The samples may be shared with other researchers, if the Research Ethics Committee approves this.

7. What do I have to do?
You don't have to do anything, apart from to give your consent. The tissue will be taken to the laboratory and we will do the tests there.

8. Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

9. What are the disadvantages and risks of taking part?
The only difference to your treatment is that in some patients, a second biopsy will be taken, and that a blood sample will be taken on each occasion. There is a small risk of causing an adhesion at the biopsy area in the eye. This risk is greater when the eye is red and inflamed.

10. What are the possible benefits of taking part?
By taking part and allowing us to use your cells in this way, we hope that you will be helping to improve our understanding of and find new treatment for ocular pemphigoid, which may in the long term benefit you and other patients with this disease.

11. What happens when the research study stops?
The information we gain and any remaining tissue samples will be stored for up to 10 years.

12. What if something goes wrong?
It is difficult to foresee any harm resulting from taking part in this study. If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

13. Will my taking part in this study be kept confidential?
If you agree, your GP will be informed of your participation in this research.
None of the information related to the tissue will be traceable to you, as all personal information will be removed.

14. What will happen to the results of the research study?
The results of this study are will be published in a number of scientific journals and in Dr Saw's Doctorate of Philosophy thesis. You will not be identifiable from any published results of the study if you request this on the consent form.

15. Who has reviewed the study?
The Moorfields & Whittington Local Research Ethics Committee (Ref 06/Q0504/20) has reviewed this study.

16. Contacts for further information

Miss Suzanne Cabral, Research Co-ordinator	Tel: 020 7566 2036
Miss Valerie Saw, Corneal Fellow	Tel: 020 7566 2320
Mr John Dart, Consultant Ophthalmic Surgeon	Tel: 020 7566 2320

THANK YOU

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Moorfields Eye Hospital NHS
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THANK YOU

You may keep a copy of this information sheet and the consent form you have signed.

C. Consent Forms

Moorfields Eye Hospital NHS
NHS Foundation Trust

Moorfields & Whittington LREC 06/Q0504/20
Patient Identification Number:

City Road
London
EC1V 2PD

CONSENT FORM- Active Pemphigoid

Tel: 020 7253 3411
www.moorfields.nhs.uk

Title of Project: Conjunctival scarring in ocular pemphigoid

Name of Researchers: Miss Valerie Saw, Mr John Dart, Dr Virginia Calder, Dr Julie Daniels

1. I confirm that I have read the attached information sheet on the above project, dated 10th April 2006 (Version 1) for the above study and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved. ☐

2. I agree to give samples of conjunctiva and blood for research in the above project. I understand how the samples will be collected, that giving a sample for this research is voluntary and I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐

3. I understand that if I agree, my GP will be informed of my participation in this project ☐

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

5. I agree that the samples I have given can be looked after and stored at the Institute of Ophthalmology, as described in the attached information sheet. I understand that researchers other than those listed above may carry out some of these projects. ☐

6. I know how to contact the research team if I need to: Miss Suzanne Cabral, Research Co-ordinator, telephone number 020 7566 2036. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Would you like to be sent information about the results of the research? YES ☐ NO ☐

Thank you for giving samples for research

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Chairman: Sir Thomas Boyd-Carpenter
Chief Executive: Ian Balmer
Patron: Her Majesty The Queen

Pemphigoid Lab Research Consent- Active v1 10.04.2006 no highlight.doc

Moorfields Eye Hospital NHS
NHS Foundation Trust

Moorfields & Whittington LREC 06/Q0504/20
Patient Identification Number:

City Road
London
EC1V 2PD

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CONSENT FORM- Controlled Pemphigoid

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Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Would you like to be sent information about the results of the research? YES ☐ NO ☐

Thank you for giving samples for research

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 6: Investigating Fibroblast and Epithelial cell markers

A. Cytokeratin as an epithelial cell marker

Anti-pan-cytokeratin (Sigma) was initially evaluated as an epithelial cell marker, and CD44, a transmembrane protein present on the surface of most vertebrate cells which binds to the extracellular matrix protein hyaluronan, was used as a label for both the epithelial cells and fibroblasts. However, cultured human fibroblasts can express cytokeratins 5, 8, 14, 16 and 17 (Katagata *et al.*, 2002), and it was found that over 90% of cultured conjunctival fibroblasts stained positively with the cytokeratin marker (see **Figure 1**). An additional disadvantage to using cytokeratin to exclude epithelial cells was that it is an intracellular protein and the cells had to be fixed and permeabilised for cytokeratin staining; the cells could not be sorted on a cell sorter and then used for further experiments as the fixation process would render the cells non-viable.

Some groups have judged the purity of conjunctival fibroblast cultures based on both cell morphology and positive staining with vimentin and negative staining with cytokeratins (Fujitsu *et al.*, 2003; Leonardi *et al.*, 2002). However vimentin, an intracellular filament protein, can also be expressed in epithelial cells.

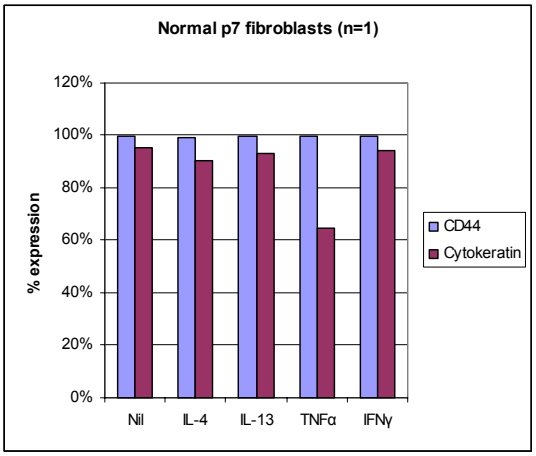


Figure 1. CD44 and cytokeratin expression in normal passage 7 (p7) conjunctival fibroblasts, in response to 16 hours of cytokine stimulation

B. Thy-1 and FSP1 as fibroblast markers, and CD326 as an epithelial cell marker

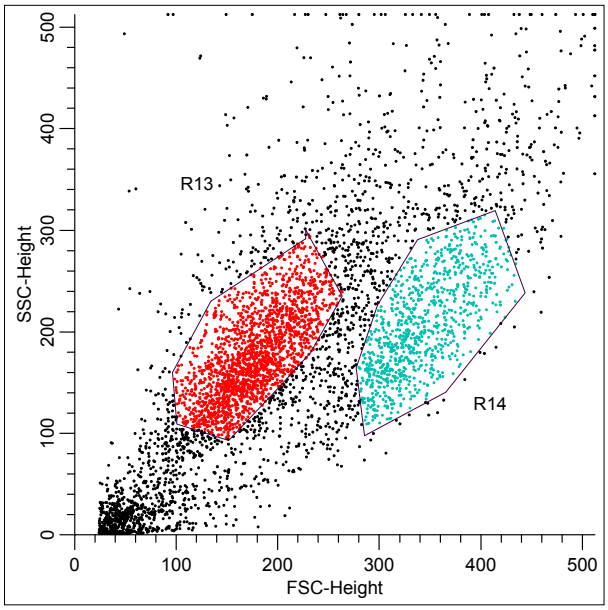
Thy-1 (CD90) and fibroblast surface protein-1 [1B10] as fibroblast markers were investigated on 2 fibroblast cell lines, human corneal fibroblasts and human embryonic lung fibroblasts. Thy-1 (also known as CD90 or AS02) has been frequently used as a fibroblast surface marker (Puxeddu *et al.*, 2006), as its expression is restricted to fibroblasts, neurons and some CD34+ blood stem cells. However, it has been reported that loss of fibroblast Thy-1 expression correlates with increased fibrogenesis in the lung (Hagood *et al.*, 2005), so its value as a fibroblast marker in fibrogenic diseases is questionable. Fibroblast surface protein [1B10] is present on fibroblasts, smooth muscle differentiated fibroblasts, tissue macrophages and 95% of peripheral blood monocytes, but is absent on human epithelial cells, lymphocytes and vascular smooth muscle cells (Ronnov-Jessen *et al.*, 1992; SundarRaj *et al.*, 1984).

CD326 (also known as EpCAM, epithelial cell adhesion molecule) is a transmembrane glycoprotein involved in cell adhesion, which is expressed on most epithelial cells and carcinomas, but is not found on normal fibroblasts.

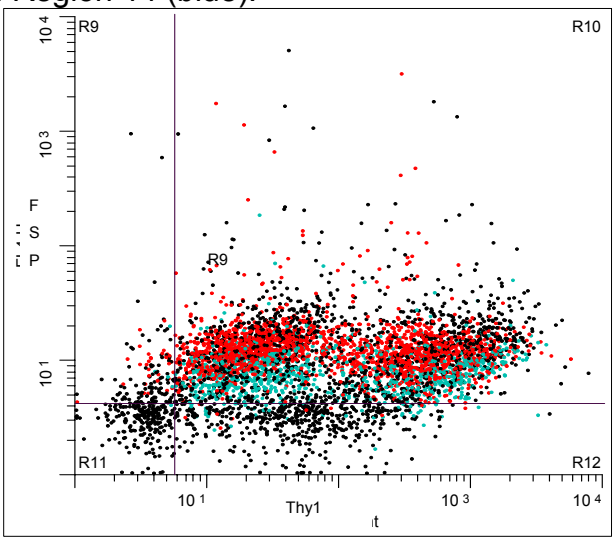
C. Flow cytometry of human corneal fibroblasts and human embryonic lung fibroblasts

Almost all (97%) the passage 6 human corneal fibroblasts tested showed both Thy-1 and FSP staining, and were CD326 negative (**Figure 2**). However only 77% were CD44 positive. These experiments need to be repeated to confirm the results.

Figure 2. Thy-1, FSP, CD326 and CD44 expression by passage 6 human corneal fibroblasts

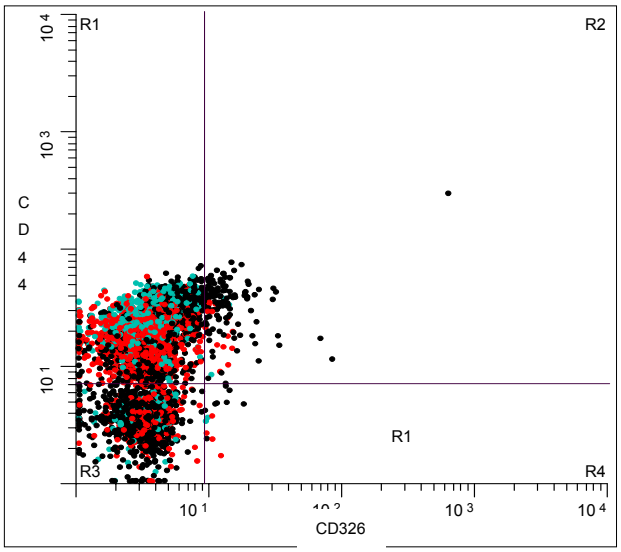


a. Forward scatter versus side scatter showing 2 populations of cells, gated as Region 13 (red) and Region 14 (blue).



b. Thy-1 expression versus FSP expression.

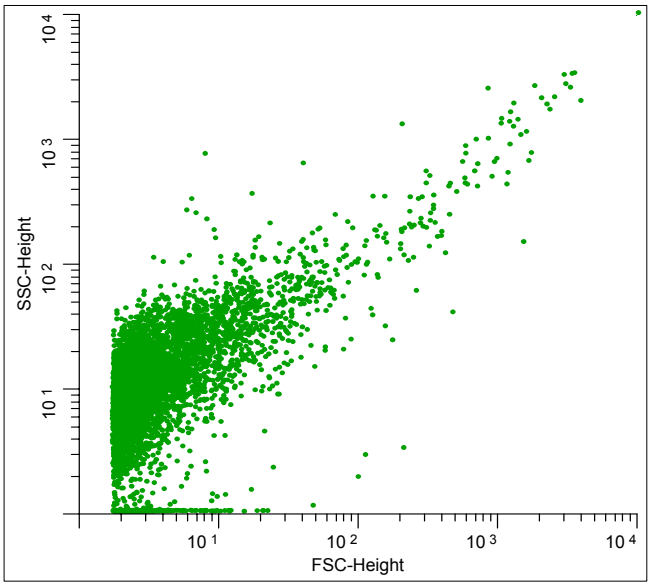
The majority of cells in both Region 13 and Region 14 cells are both FSP positive and Thy-1 positive. R13 cells show greater intensity FSP staining. Within both R13 and R14 there are 2 populations of cells with differing intensities of Thy-1 staining.



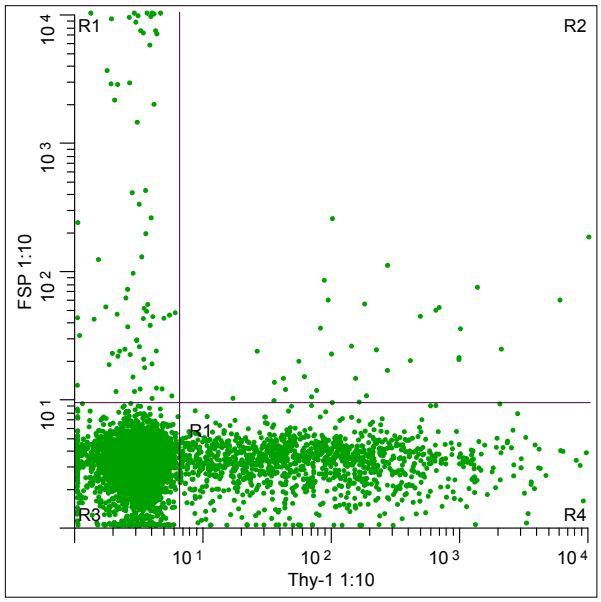
c. CD326 expression versus CD44 expression.
Almost all cells are CD326 negative (97%). 77.33% of all cells are CD44 positive; almost all Region 14 cells are CD44 positive, but a proportion of Region 13 cells are CD44 negative.

Human embryonic lung fibroblasts did not express FSP and only 26% expressed Thy-1 (**Figure 3**). CD326 did not appear to be expressed by these fibroblasts, and CD44 expression was only 12%. It may be that these embryonic cells are not differentiated enough to express these fibroblast markers. These preliminary findings need to be repeated to confirm the results.

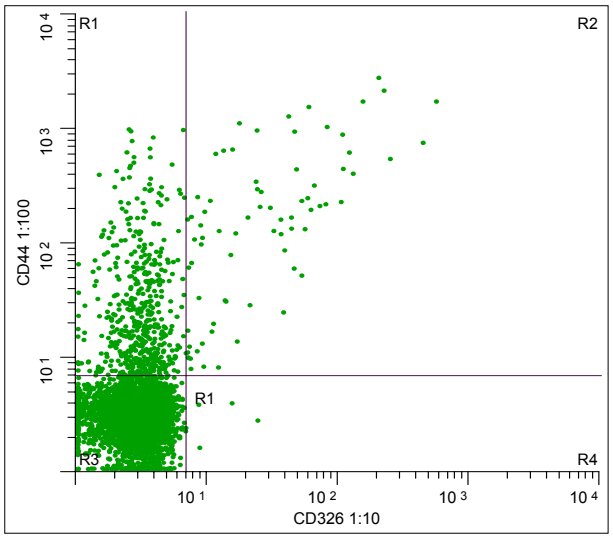
Figure 3. Thy-1, FSP, CD326 and CD44 expression by human embryonic lung fibroblasts



a. Forward scatter versus side scatter



b. Thy-1 expression versus FSP expression: Thy-1 expression 26% and FSP expression 2%



c. CD326 expression versus CD44 expression: CD326 expression 1% and CD44 expression 12%

In summary, FSP may be a suitable fibroblast surface marker, and an alternative to Thy-1. CD326 does not appear to be expressed on the two types of fibroblasts tested. Along with confirmation of the results by repeating the above experiments, the next step would be to evaluate CD326 expression in an epithelial cell line, and to show that by mixing both a fibroblast cell population and an epithelial cell population, that there is specific staining for each cell type, which can identify the two populations. These markers could then be tested on the explant-derived human conjunctival fibroblasts to show reduction in the proportion of epithelial cells with increasing passage number.